



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

In Re Application of: James P. Elia)	
)	Group Art Unit: 1647
Serial No.: 09/064,000)	
)	Examiner: Daniel C. Garnett
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	Client Docket No. Case No. 1
OF SOFT TISSUE)	

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APPEAL BRIEF

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REAL PARTY IN INTEREST

The real parties in interest in the instant appeal are Assignees, Dental Marketing Specialists, Inc., an Arizona corporation, 9377 E. Bell Road, Suite 385 Scottsdale, Arizona 85260, and Jerry W. Bains and Salee C. Bains Irrevocable Trust, 9013 Red Lawrence Drive, Carefree, Arizona 85377. Subsequent to the assignment recordal for the instant application, the address of Dental Marketing Specialists, Inc. changed to 7364 East Crimson Sky Trail, Scottsdale, Arizona 85262. Also, subsequent to the assignment recordal for the instant application, the address of Jerry W. Bains and Salee C. Bains Irrevocable Trust changed to 39096 N. 102nd Way, Scottsdale, Arizona 85262.

RELATED PROCEEDINGS

There are no related appeals, interferences, or judicial proceedings known to Appellant, Appellants' legal representative, or Assignee, which may be related to, directly affect, be directly affected by, or may have a bearing on the Board's decision in the pending appeal, except for the following:

1. Appellant's Appeal Brief filed August 28, 2008, in co-pending application Serial No. 10/179,589;
2. Notice of Appeal mailed to the Patent and Trademark Office (hereinafter "PTO") on September 30, 2008, in co-pending application Serial No. 09/794,456; and
3. Appellant's Reply Brief filed on March 18, 2008, in co-pending application Serial No. 09/836,750. A Request for Continued Examination (hereinafter "RCE") was also filed on June 6, 2008, in co-pending application Serial No. 09/836,750 to ensure the entry and consideration of additional evidence. As of the present date, the PTO has not acted upon the RCE.

The attached Related Proceedings Appendix confirms such statement.

STATUS OF CLAIMS AND CLAIMS UNDER APPEAL

Claims 1-6 were cancelled in the Amendment filed February 15, 2001.

Claims 7-191 were cancelled in the Amendment filed September 3, 2002.

Claims 192-381 were cancelled in the Amendment filed August 19, 2004.

Claims 382-402 and 406 were cancelled in the Amendment filed November 28, 2007.

In view of the above-identified cancellation of claims 1-402 and 406, the correctness of the May 5, 2008, Final Rejection (hereinafter "Final") made by the PTO of claims 403-405 and 407-412 is being appealed. Specifically, the instant appeal includes the rejection of claims 403-405 and 407-412 under 35 U.S.C. §112, second paragraph, as being indefinite; the rejection of claim 404 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement; the rejection of claims 403-405 and 407-412 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement; the provisional rejection of claims 403-405 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163 and 170-173 of co-pending application Serial No. 10/179,589; and the new provisional rejection of claims 403 and 407-412 under 35 U.S.C. §101, as claiming the same invention as that of claims 161-164 and 172-174 of co-pending application Serial No. 10/179,589.

STATUS OF AMENDMENTS

No amendment has been made or entered subsequent to the Final.

SUMMARY OF CLAIMED SUBJECT MATTER

Appellant's invention is directed to a method of growing and integrating a desired artery at a selected site in the body of a human patient. Claim 403, the only independent claim on appeal recites a method comprising locally injecting stem cells in the body at a selected site, forming a bud (primordium) at said site and growing an artery from said bud. This method is described in the instant specification at page 20, lines 10 thru page 21, line 15; page 30, line 14 thru page 32, line 19; page 33, lines 8–10; page 37, lines 19–25; pages 40–42 and 44–48 and 52. Claim 404 depends from claim 403 by requiring growth of an artery in the leg of a patient by intramuscular injection of stem cells. Intramuscular injection to grow an artery in a patient's leg is described on pages 45, and in Example 18. Claim 407 depends from claim 403 and defines the stem cell as being harvested from bone marrow; claim 408 depends from claim 407 and defines obtaining bone marrow from the patient; claim 409 depends from 403 and requires using bone marrow obtained from peripheral blood; and claim 410 depends from claim 409 and requires using the patient's blood. The specification describes the subject matter of claims 407–410 at pages 40–42, and pages 47–52 wherein the specification discloses using a patient's own stem cells for growing multiple described organ species, through differentiation and morphogenesis. The organ species artery is specifically disclosed as a desired target organ on page 52. Additionally, pluripotent stem cells are described at page 50. Claims 411 and 412 define determining blood flow through the artery and observing the grown artery. The specification discloses such procedures in Examples 18 and 19, pages 54, and 56.

GROUND OF REJECTION FOR REVIEW ON APPEAL

Claims 403-405 and 407-412 were finally rejected under 35 U.S.C. §112, second paragraph, as being indefinite.

Claim 404 was finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

Claims 403-405 and 407-412 were finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

Claims 403-405 were provisionally finally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163 and 170-173 of co-pending application Serial No. 10/179,589.

Claims 403 and 407-412 were newly provisionally finally rejected under 35 U.S.C. §101, as claiming the same invention as that of claims 161-164 and 172-174 of co-pending application Serial No. 10/179,589.

ARGUMENT

Rejection under 35 U.S.C. §112, Second Paragraph – Indefiniteness

Claims 403-405 and 407-412 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The Examiner considers these claims to be indefinite because it is unclear whether the step of “forming a bud” is an intrinsic step of artery formation or whether the practitioner must perform further action to form an artery. Applicant disagrees that claims 403-405 and 407-412 define subject matter that fail to meet the definiteness requirements of 35 U.S.C. §112, second paragraph. Appellant herein argues the patentability of each claim.

The Examiner questioned whether the formation of a bud would be an intrinsic step in artery formation or whether the practitioner would require further action. Examiner seems to be confusing the “definiteness” requirement of the second paragraph with the theory underlying Applicant’s invention. The Examiner has not explained how an understanding of the underlying theory of the invention is required to render the claimed subject matter definite to one skilled in the medical art. Rather, it is clear from the specification that the only step required by the practitioner is that of injecting stem cells into a selected site in a patient’s body. Once injected, the stem cells interact with the human host by differentiating along predetermined physiological developmental pathways to form a vascular bud which grows into an artery. One skilled in the medical art would clearly understand and appreciate that organs, such as arteries, would grow in the body of a human patient from a bud primordium without further action by the practitioner. One skilled in the medical art reading the claims in this light would clearly understand their scope. Accordingly, Appellant does not believe that it is necessary to

amend the claims as suggested by the Examiner at page 3 of the Final to overcome this rejection. However, should the Board believe that such amendment is required to overcome the rejection, Applicant stands ready to make the suggested amendment.

Rejection under 35 U.S.C. §112, First Paragraph - Description

Claim 404 stands rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner states that the specification, as originally filed, does not provide antecedent support for language calling for the “administration of cells to a damaged site in a leg of a patient.” Applicant disagrees.

On page 12, ¶15 of the Final, the Examiner correctly frames the lack of description issue as bottomed on an inquiry as to whether the instant specification taken as a whole would reasonably lead one skilled in the art to practice the method defined by claim 404. What the Examiner apparently fails to appreciate, however, is that such an inquiry is a question of fact. In re Wertheim, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). Based on this record, it is patently clear that the Examiner’s rejection lacks a sound factual basis.

Example 18 provides a written description of intramuscular administration of cDNA clones encoding VEGF into ischemic tissue (damaged artery) in the leg of a human patient to promote artery growth. The Examiner’s prior statement that, “a damaged artery in a leg is not the same scope as damaged site in a leg” is inept at best. The real issue is not whether the language “damaged site” is specifically recited in Example 18 but whether the concept of administering a soft tissue promoter to a

damaged site in the leg of a patient is conveyed by the original disclosure considered as a whole. See In re Anderson, 471 F. 2d 1237, 176 USPQ 331, (CCPA 1973) and In re Rasmussen, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981). The specification as filed is replete with disclosure relating to the concept of administering compositions to a “desired site” in the body for promoting the growth of soft tissue, such as an artery, as described in Example 18 and on page 53, lines 20-21 of the specification, which clearly teaches (page 53, lines 20-21) that, “the selection of sites can vary as desired.”

Applicant further disagrees with the Examiner’s statement that, “the specification does not envision administration of cells at the damaged artery.” The written description requirement of the statute “serves to insure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter later claimed by him; how the specification accomplishes this is not material.” In re Wertheim, supra. The court in In re Alton, 76 F.3d 1168, 37 USPQ 1578 (Fed. Cir. 1996) held that an applicant in satisfying the written description requirement:

“...does not have to utilize any particular form of disclosure to describe the subject matter claimed, but ‘the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.’ In re Gosteli, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989) (citation omitted). Put another way, ‘the applicant must . . . convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention.’ Vas-Cath, 935 F.2d at 1563-64, 19 USPQ2d at 1117. Finally, we have stated that ‘[p]recisely how close the original description must come to comply with the description requirement of section 112 must be determined on a case-by-case basis.’ Eiselstein v. Frank, 52 F.3d 1035, 1039, 34 USPQ2d 1467, 1470 (Fed. Cir. 1995) (quoting Vas-Cath, 935 F.2d at 1561, 19 USPQ2d at 1116).”

It is trite law that the Examiner, when determining compliance with the description requirement of the statute, must consider the entire disclosure as it would be understood by one skilled in the art at the time the application was filed. , In re Anderson, supra. The Examiner's statement at page 10, ¶9 of the Final that:

Therefore, for one of skill in the art to even think of extrapolating example 18 to guide the use of cells, the skilled artisan would already have to know the very thing that Applicant claims to have been the first to discover...

evinces a classic failure on the part of the PTO to meet its initial burden of presenting evidence why persons skilled in the art would not perceive in Appellant's specification a reasonable description of the subject matter defined in the claim in issue. Indeed, the contemporary work described in the U.S. Patent No. 5, 980,887 to Isner et al. (of record and hereinafter referred to as "Isner '887") and in the Asahara et al. publication¹ (hereinafter referred to as "Asahara") is evidence that the skilled artisan was aware at the time of filing of Appellant's application that cell therapy using intramuscular injection of endothelial progenitor stem cells was known for treating ischemic tissue in a human patient as an alternative to intramuscular injection of DNA encoding VEGF. Isner '887 clearly discloses that cells and genes are alternatives in the Summary of the Invention at column 3, lines 1-18. The Examiner's statement at page 11, ¶12 of the Final that:

Rather, Applicant relies on the notion that a teaching for cDNA for growing an artery intrinsically and inherently causes one skilled in the art to envision using cells for the same purpose

¹ Science, 275:964-967 (1997). The Science article is a publication of the work disclosed and claimed in Isner '887 and is cited in the Isner '887, a copy of which is attached hereto for the readers' convenience as Exhibit A.

clearly misstates Appellant's position. On what basis does the Examiner make such a clearly erroneous assertion? What Appellant argues is that one skilled in the art possessing an understanding of the contemporary prior art, such as evidenced by the Isner '887 patent, when reading the instant specification would understand that its context clearly describes that cDNA and stem cells when injected intramuscularly in a human patient are alternative compositions for providing ischemic tissue with increased blood vessels – that Appellant was clearly in possession of the claimed subject matter at the time of filing of the instant application.

The Examiner has failed to explain where the language of claim 404 calling for “injecting stem cells...at a damaged site” in a patient's leg defines subject matter completely outside the scope of Appellant's specification. The specification clearly contains a description of the claimed invention using descriptive words that fully set forth the claimed subject matter, albeit not *in haec verba*. See Eiselstein v. Frank, 52 F.3d 1035, 1038, 34 USPQ 2d 1467, 1470 (Fed. Cir.1995).

Applicant submits that all the limitations of claim 404 appear in the specification as originally filed. The specification, starting at page 20, line 10, defines growth factors as compositions that promote soft and hard tissue growth. The specification is replete with description of inserting a soft tissue growth promoter at a desired (damaged) site in the body (pages 10, 18, 20, 21, 31, 32, 45, 52, 53, 56, and 62). Appropriate compositions which promote the growth of soft tissue within the scope of Applicant's invention are described as comprising a patient's own cells (pages 47 and 48) and particularly stem cells (pages 37, 40, 41, 42, 48, 51 etc.) including autologous and allogeneic global bone marrow stem cells (bone marrow mononuclear cells/BMCs) and adult stem cells

collected from peripheral blood. One skilled in the art reading the subject application would understand that Applicant's invention is not limited to using a particular soft tissue promoter, such as the cDNA encoding VEGF specifically described in Example 18 but, rather, includes the use of a broad class of described soft tissue promoters, including cells, such as stem cells. Claim 404 is directed to an alternative embodiment (elected invention) to the soft tissue promoter delineated in Example 18. One skilled in the art reading the subject matter disclosed on page 47, lines 22 through page 48, line 15 of the instant specification would readily understand that, as of the filing date, Applicant was in possession of the concept of employing a patient's own stem cells to promote the growth of an artery within the scope of claim 404.

It is noted that the Examiner no longer asserts that Appellant's teaching of, "administration to arterial walls...as described in Example 18]...does not support the recitation of intramuscular injection" and for good reason. Reference to any common medical dictionary confirms that arteries have a muscle component.

The Examiner acknowledges that the instant specification teaches that cells are included in the class of soft tissue growth promoters. See, in particular, page 7, ¶14, of the July 24, 2007 Office communication where the Examiner states, "Therefore, *in the lexicon of this specification*, 'cells' may be a subgenus of 'growth factor'." Paragraph 4 of the Third and Fourth Supplemental Declarations of Drs. Heuser and Lorincz (of record) confirm that one skilled in the art to which the invention is directed would reach the same conclusion when reading the instant specification. The Examiner's reading and acknowledgement of the content of the specification is consistent with the mandate of

the *en banc* CAFC decision in Phillips v. AWH Corporation, 415 F.3d 1303 (Fed. Cir.2005).

Appellant believes that the Examiner's acknowledgement that cells are growth factors establishes a material fact in this record which is the law of the case. In related application Serial No. 09/794,456, Examiner Kemmerer reached the same conclusion at page 6, lines 1-8 in the February 22, 2006 PTO communication. A copy of such portion of the PTO communication is attached hereto as Exhibit B for the readers' convenience. Perforce, this established material fact requires the Examiner to consider all relevant portions of Appellant's disclosure in evaluating the Section 112 "description" issue herein, including disclosures related to the genus "growth factor." In view of this material fact, it was error for the Examiner to fail to consider the original disclosure as a whole, i.e., the above-mentioned genus and species relationship, when determining the specification's compliance or non-compliance with the description requirement of 35 U.S.C. §112, first paragraph. Thus, the Examiner's position disregards the tenants of relevant case law such as In re Anderson, supra; In re Rasmussen, supra; and Johnson and Farnham, 558 F.2d 1008, 194 USPQ 187, 195 (CCPA 1977).

The Examiner at page 10, ¶10 of the Final states that, "Each patent application is evaluated on its own merits." Of course this is true. The Examiner has proffered no objective evidence that stem cells and cDNA clones function differently in the context of Appellant's invention, and for good reason. Indeed, the record, as evidenced by Isner '887 and Asahara, establishes as a material fact that they possess a common functionality - they belong to a class of compositions that promote growth of soft tissues (blood vessels) in a human patient. Further, Nabel U.S. Patent No. 5,328,470 (of record and

hereinafter “Nabel”) provides further evidence that those skilled in the art at the time of filing of Appellant’s application were aware of the alternative use of cells and DNA vector in the site-specific treatment of cardiovascular diseases, including the perfusion of ischemic tissue. Nabel clearly teaches that cells or appropriate vector (DNA) can be “surgically, percutaneously, or intravenously” introduced into the patient. The Examiner’s statement on page 11, ¶13 of the Final that Appellant’s citation “is inexplicable” is indeed puzzling. One skilled in the art reading Nabel would clearly understand that this patent teaches more than a kit comprising DNA. Nabel, Isner ‘887, and Asahara are evidence of the state of the art at the time of filing of the instant application.

The record (Law) of this case establishes that the PTO in requiring an election of species in the instant application and in co-pending and related application Serial Nos. 09/794,456 and 09/836,750 has held cells (stem cells) to be a species within the disclosed class of growth promoters (growth factors). The Examiner’s reliance on case law relating to genus-species requirements misses the point. The present Examiner is bound by prior PTO holdings. It is error for the Examiner at this late stage of prosecution to contend otherwise.

Moreover, the PTO’s species election requirement is consistent with the issuance of Isner ‘887 and Nabel, both of which treat cells and genes (DNA vector) as alternative agents for promoting the growth of blood vessels in ischemic tissue. This is contrary to the Examiner’s erroneous assertion of lack of functionality. It is further pointed out that the scope of claims issued by the PTO for Isner ‘887 encompass “VEGF cDNA” and “cells” as alternative angiogenetic promoters, i.e., capillary blood vessel promoters, not

distinct inventions. Isner '887 differs from the present invention by disclosing and claiming injecting endothelial progenitor cells that are necessarily limited to promoting endothelial cell growth (capillary blood vessels), not artery growth as required in instant claim 404.

Further, the PTO's issuance of the U.S. Patent No. 7,097,832 to Kornowski et al. (of record and hereinafter "Kornowski"), in the same Class 424/93.7 as Isner '887, evidences that the Isner '887's use of endothelial progenitor cells promotes capillary blood vessel growth, not artery growth. This fact is also confirmed in a post-filing date article published by the American Heart Association entitled, "Endothelial Progenitor Cells: More Than an Inflammatory Response" (of record). What the Examiner has failed to appreciate is that, on this record, Dr. Elia was the first to recognize that cDNA encoding VEGF and stem cells are alternative soft tissue growth promoters for growing arteries by direct intramuscular injection into a human patient. This is a material fact established on this record regardless of Appellant's manner of "reduction of practice" for the present invention.

There can be no doubt that under current law the instant specification fairly satisfies the description requirement of the statute by containing a reasonably equivalent description of the subject matter called for by claim 404. The language of claim 404 added by the Amendment filed November 3, 2006 finds antecedent support in the disclosure of the application as filed and, perforce does not constitute new matter.

Rejection under 35 U.S.C. § 112, First Paragraph – Enablement

Claims 403-406 and 407-412 stand finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Appellant disagrees that the subject specification fails to enable the claimed subject matter under current law. Appellant herein argues the patentability of each claim.

It is well settled that enablement issues are determined by consideration of an applicant's specification along with knowledge in the art at the time of filing, United States v. Teletronics, 857 F. 2d 778, 785; 8 USPQ 2d 1217, 1223 (Fed. Cir.1988, *cert. denied* 490 U.S. 1946 (1989)). Appellant submits that the instant specification, when considered in view of the knowledge in the art at the time the application was filed, enables one skilled in the medical art to make and use the claimed invention.

Appellant submits that are three major points to consider when determining whether the instant specification contains a disclosure that would have enabled a skilled person in the medical art to make and use the claimed invention within the purview of the statute. The points are: 1) the specification disclosure; 2) the knowledge in the art at the time the application was filed; and 3) the skill level in the art. When these points are considered, there should be no doubt that Appellant's specification provides an enabling disclosure.

First, there is a considerable body of disclosure provided by the subject application relating to Appellant's disclosed invention of promoting the growth of soft or hard tissue in human patients—including growing a new artery as called for by the claims at issue — by administering a broad class of growth factors, including stem cells, suitable for affecting such tissue growth. In this regard, Appellant's specification (pages 10, 20,

21, 30-33, and 37-52) provides a substantial body of disclosure regarding using a growth factor to form a bud and thus grow soft tissue in a human body. These portions of the specification describe a class of claimed and unclaimed growth factors that broadly and specifically include genes, nucleic acids, a patient's own cells (autologous cells), or universal cells, e.g., stem cells (global mononuclear bone marrow cells), etc., all of which are described to promote tissue growth through differentiation and morphogenesis. Appellant's broad and specific disclosure relating to the aforementioned class of growth factors patently provides a scope of enablement which includes stem cells broadly (pages 37, 48, 50, and 51) and bone marrow mononuclear stem cells specifically (pages 40-42). Such disclosure is commensurate in scope with the subject matter of the claims at issue.

Second, the record clearly establishes that the administration techniques, apparatus, and administered compositions disclosed and claimed by Appellant were old and well known as of the filing date of the instant patent application.

Isner '887 and Asahara constitute contemporary prior art knowledge which employed a limited subpopulation of EC progenitor stem cells isolated from human peripheral blood for promoting capillary growth. Isner '887 and Asahara evidence that those skilled in the art prior to the 1998 filing date of Appellant were aware that EC progenitor cells (stem cells) and DNA encoding VEGF are alternatives for treating blood vessel injuries, i.e., ischemic tissue. Isner '887 at column 7, lines 17-23 of the patent, discloses that "any suitable means" can be used to administer stem cells, including intramuscular injection. Nabel teaches one skilled in the art that cells and genes can be either locally (injection) or systemically administered to human patients to treat organs affected by disease, including ischemic tissue. Although these patents are directed to

different inventions than that of Appellants, i.e., employ different cells and achieve different results, they nevertheless apprise one skilled in the art of prior art methods commonly used for administering genes and cells for the treatment of human diseases involving ischemic tissue. Such objective evidence must be taken into consideration by the PTO when determining enablement under 35 U.S.C. §112, first paragraph.

One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or blood of the patient would understand that the claimed invention distinguishes from Isner '887 by describing using unfractionated (global) bone marrow mononuclear cells. There is no basis in fact for determining that a fractionated population, such as EC progenitor cells, is disclosed by Appellant because there is no disclosure that the harvested cells are separated and then a separated portion administered to a patient. Reading the disclosure otherwise distorts the reasonable/intended reading of Appellant's specification. Isner '887 serves to apprise one skilled in the art of general methods for implanting endothelial progenitor stem cells for forming capillary blood vessels. One skilled in the art being so apprised and reading the instant specification would understand that Appellant has provided sufficient information, i.e., the process steps and ingredients essential to grow an artery as set forth in the claims.

Further evidence supporting enablement may be found in the form of the February 13, 2001 Declaration of Dr. G. Robert Meger (of record) which demonstrates that the disclosed and claimed administration techniques used in practicing the invention were known at the filing date of the application. The administration techniques disclosed by Appellant were routinely employed in the medical art, but not in the claimed combination with the claimed materials, at the time the instant application was filed. See

in particular the discussion in Isner '887 and Asahara in regard to the medical art's prior use of bone marrow transplants (HSCs) in treating diseases. Isner '887 acknowledges using techniques similar to those used in the medical arts for recovering HSCs in obtaining endothelial progenitor cells (CD34+). The collection, handling, and reimplantation of HSCs are so well known and notorious in the medical arts that the Board should take Official Notice of same.

In any event, Appellant submits that such disclosure of the instant specification and existing knowledge in the art such as that identified by Dr. Meger, as well as the work of Isner '887, Asahara, and Nabel, would enable a skilled practitioner to practice the claimed invention. As will become evident later, two experts in the medical field, Drs. Richard Heuser and Andrew E. Lorincz, being apprised of relevant portions of Appellant's specification, confirm such conclusion.

Third, the PTO has acknowledged that the level of skill in the medical art is high. Appellant agrees that the skill level is high when it is considered that many years of education, training, and experience are required in the medical field. The instant specification is addressed to and is understood by such highly skilled persons.

Once the above-identified relevant materials and administration techniques set forth in the subject specification are properly considered in their entirety, Appellant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced, as noted above, by the fact that the materials and administration techniques, but not the inventive results, were well known when the instant application was filed. MPEP Section 2164 states that the purpose of the enablement requirement is to describe the claimed invention in such

terms to permit one skilled in the art to make and use the invention. Such Section cautions that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention. For the reader's convenience, MPEP Section 2164.01 states that:

A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F2d. 660, 661, 18 USPQ 2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F2d. 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) cert denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Co.*, 730 F2d. 1452, 1463, 221 USPQ 481, 489 (Fed. Cir.1984).

Appellant believes that the above caution is especially relevant to the instant factual situation where the Examiner has conceded that there was a high level of skill in the art at the time the instant application was filed and, further in view of the evidence contained in Isner '887, Asahara, Nabel, and Dr. Meger's Declaration that the methods and apparatus needed to practice the invention were well known at the time of the invention. Thus, Appellant submits that the instant disclosure clearly enables one skilled in the medical arts to make and/or use the full scope of the claimed invention without undue experimentation. A reasonable consideration of the three above-delineated points and the interaction thereof compels such conclusion.

Appellant's above conclusion that one skilled in the art is enabled to make and use the claimed invention is consistent with the Examiner's acknowledgement at page 14, ¶18 of the Final that the state of the art after Isner '887 disclosure was such that Appellant's claimed "...method was known to be possible." Accordingly, the

enablement issue should be put to rest because the Isner '887 and Asahara disclosures are prior to or contemporary with the filing date of the instant application.

The Examiner has the burden to establish and support by convincing objective evidence a *prima facie* case of lack of enablement. For reasons set forth below, Appellant believes the Examiner has failed to meet such burden.

The first paragraph of the statute requires nothing more than objective enablement, and it is of no importance whether such teaching is set forth by use of illustrative examples or by broad terminology. As a general matter, an application's disclosure, which contains a teaching of how to make and use the invention in terms which correspond in scope to those used in describing the invention sought to be patented, is considered to be in compliance with the enabling requirement of the statute. In re Marzocchi, 439 F.2d 220, 169 USPQ 367, 369-370 (CCPA, 1971). Further, "Section 112 does not require that a specification convince persons skilled in the art that the assertions therein are correct." [Emphasis added]. In re Robins, 429 F.2d 452, 166 USPQ 552 (CCPA, 1970).

When evaluating enablement, it is incumbent upon the Examiner to determine what subject matter each claim recites, i.e., the scope of protection sought for each claim. The scope of dependent claims are properly determined with respect to 35 U.S.C. §112, fourth paragraph. See MPEP Section 2164.08. It is submitted that the Examiner has failed to perform such required analysis. Appellant notes that the Examiner has not addressed the subject matter of each claim separately. Appellant has argued that the subject matter of all claims finds enabling support in the specification.

Appellant further points out that it is evident the Examiner failed to consider the disclosure provided by the subject specification as a whole in determining compliance with the enablement requirement of the statute. The appropriate factual determination is whether the instant specification reasonably directs one skilled in the art how to make and use the claimed subject matter. As demonstrated above, the Examiner erroneously restricted the factual determination to the elected species of growth factor and, thusly, ignored those portions of the specification describing a broader generic invention and also ignored disclosure related to non-elected species. Appellant is entitled to have the entire disclosure considered in determining compliance with 35 U.S.C. §112, first paragraph. See In re Anderson, supra and In re Johnson and Farnham, supra and such determination must take into consideration that which is known in the prior art – that a patent should preferably omit that which is well known/understood in the particular art to which the claims are directed.

Turning to the reasons proffered by the Examiner regarding non-enablement, Appellant presents the following remarks.

The Examiner at pages 15-17, ¶20 of the Final asserts that the instant specification fails to provide any guidance as to how to use any kind of cell , much less a stem cell, to grow an artery. The disclosure at page 47, line 22 through page 48, line 15 of the specification clearly rebuts the Examiner's notion that Appellant never clearly enunciated using stem cells (bone marrow stem cells) for promoting direct differentiation and morphogenesis into an organ. Of course, one skilled in the art would recognize that growth of an organ encompasses an artery. Page 45 of the specification sets forth the well recognized medical fact that "[a]n artery is an organ from the circulatory system."

Examiner's statement that the specification fails to "provide any guidance as to how to use stem cells to grow an artery evinces a lack of understanding of how differentiation and morphogenesis occurs *in vivo*. The fact that stem cells home to foci of ischemic tissue was known to those skilled in the art at the time of filing of the instant specification, as evidenced by Asahara. Hubris aside, the rhetorical theme employed by the Examiner at pages 15-17, ¶20 of the Final appears to lack proper decorum.

The Examiner at pages 17 and 18, ¶21 of the Final erroneously concludes that Appellant implicitly acknowledges that *in vivo* treatments using nucleic acids and cells had a different status in the art. Appellant has neither explicitly or implicitly, made any such acknowledgement. Appellant has continuously pointed out that Nabel and Isner '887 dispel any such notion. Both show art recognition that cells and genes comprise alternate therapeutic agents for treating human diseases, including those involving ischemic tissue. The Examiner's statement that Appellant "does not show a single organ, part of an organ, tissue, artery or even a bud by placing cells in a body" implies that enablement under the statute requires an actual reduction of practice. The Examiner has failed to cite and law or statutory/regulatory requirements that support such a contention. Appellant further notes that perhaps the Examiner is unaware that a tooth is a duplex organ comprising both hard and soft tissue.

The Examiner at page 19, ¶23 of the Final attempts to support the above position by citing authority that an actual reduction to practice is required in inventions involving "some unpredictable areas of chemistry and biology" - that conception requires an actual reduction to practice. Underlying this line of reasoning is a notion that prophetic inventions involving the medical arts cannot *ipso facto* as a matter of law satisfy the

statutory enablement requirement. The Examiner cites no regulation, statutory or case law in support of this latter reasoning. In a broader sense, the Examiner fails to appreciate that enablement is determined on a case-by-case basis which necessarily takes into consideration the state of the prior art as well as knowledge and skill possessed by workers in the art at the time of the invention. The prior art workers at the time of the present invention, as evidenced by the Asahara, understood how to locally implant (i.e., muscularly inject) endothelial progenitor stem cells (CD34+ subpopulation) into a body to locally treat ischemic tissue by growing capillary blood vessels. Appellant's contribution to the art resides in the discovery that unfractionated bone marrow stem cells, through differentiation and morphogenesis, form an organ, i.e., an artery, when locally implanted in a body. There can be no doubt that the record here when considered in its entirety compels a conclusion that Appellant's specification provides sufficient guidance for one skilled in the art to make and use the claimed invention.

The Examiner at pages 20-25, ¶¶ 24-29 of the Final attempts to explain that, while the specification may teach the concept of using bone marrow stem cells to promote the growth of an artery, there is no disclosure therein specifically defining the population or specific type of cells which would or would not grow an artery. Firstly, there is no requirement for an applicant to specify embodiments that will not work. All that is required is to specify embodiments that work. Secondly, the specification specifies using adult (autologous implantation) stem cells harvested from the bone marrow or peripheral blood of the patient. Nowhere does the specification describe using any subfraction of stem cells or even teach isolation and recovery of any subpopulation of stem cells. One skilled in the art reading the subject specification would clearly

understand that Appellant was in possession of the concept of implanting whole (unfractionated) bone marrow mononuclear cells to promote growth of organs, such as arteries, in a human patient. The Examiner correctly attributes to the Isner '887 discovery that CD34+ mononuclear cell population, present in both bone marrow and peripheral blood comprises progenitors for endothelial cells (ECs). What the Examiner fails to appreciate is that Isner '887 discloses that isolated CD34+ mononuclear cells are limited to integrating into capillary walls (column 14, lines 45-48) whereas CD34- mononuclear cells were typically found in stroma near capillaries (column 14, lines 54-57). Kornowski, in Example 3, confirms that Isner '887 forms only capillaries. Appellant's invention is directed to using unfractionated bone marrow mononuclear cells which includes CD34+, CD34-, AC133+, CD45/CD14 negative cells as well as cytokines. The claims on appeal call for a different therapeutic agent and produce a different (novel) result from the process of Isner '887.

The Examiner's charge at pages 21 and 22, ¶25 of the Final that Appellant is practicing obfuscation by taking language from different portions of the text in order to support the claimed language is disturbing indeed. Perhaps, the genesis of the Examiner's problem is the challenge of reading the specification as a whole in view of the state of the art at the time of filing. The Examiner's quote from pages 47 and 48 of the specification artfully omits the following two paragraphs:

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and

morphogenesis into an organ can occur in vivo, ex vivo,
or in vitro.

When read in its proper context by one skilled in the medical art, the language on page 48, line 13 of the specification “[i]n the example above...” refers to the formation (page 47, line 22) of “[o]rgans and/or tissues...formed utilizing the patient’s own cells.” Only an unskilled person in the medical art would be confused by the disclosures on pages 47 and 48. It appears that the Examiner, by reading the specification out of context, is employing the obfuscatory tactics that Appellant is falsely charged with.

The Examiner’s statement at pages 24 and 25, ¶29 of the Final that the specification fails to proffer any solutions to the problems encountered in regenerative medicine misses the point. The role of stem cells in the critical physiological process of angiogenesis was well known prior to both Isner ‘887 and the claimed inventions and is critical in human reproduction, development and wound repair. The specification discloses that pluripotent stem cells are required for growing multiple body parts requiring multiple tissues. As noted earlier, the Board should take Official Notice that bone marrow transplantation therapy is notoriously old. Those skilled in the art are aware that bone marrow comprises pluripotent stem cells. It is clear from Isner ‘887 that stem cells home to foci of injury. Accordingly, one skilled in the art reading the subject specification would understand that all that is required to use pluripotent stem cells to grow an artery is to implant them at the desired site.

The Examiner’s statement at page 25, ¶30 of the Final lacks merit, but none-the-less is disconcerting. Appellant has continuously argued and cited legal authority supporting the proposition that the entire specification disclosure must be considered by

the PTO when determining whether the scope of the *claimed subject matter on appeal* reasonably finds descriptive and enabling support therein. However, such argument does not open the door to the Examiner's gratuitous and sometimes derogatory expressions of opinion concerning unclaimed inventions. Perhaps, the Examiner needs to be reminded that the PTO examination process is not an adversarial proceeding.

At page 25-30, ¶¶31-36 of the Final, the Examiner addresses the conversion of gene dosages to cell dosages proffered by Appellant. Appellant disagrees with the Examiner's position. Initially, Appellant submits it is clear from MPEP Section 2164.01 (c) that it is not necessary to specify the dosage if one skilled in the art could determine such information without undue experimentation. The Examiner apparently acknowledged, at page 25, ¶31, that there is no issue regarding the absence of guidance as to how many stem cells should be used to grow an artery. While Appellant agrees that dosage is not an enablement issue, nonetheless Appellant offers the following comments in regard to the calculus employed in the conversion.

Appellant used a well established weight basis conversion method employed in the medical art for decades to convert the gene dosage of Example 18 to cells. Appellant's extrapolation was designed to demonstrate that one skilled in the medical art could easily and routinely convert the gene dosage described in Example 18 to cell dosage. The conversion is valid because one skilled in the art would reasonably understand from reading the subject specification that Appellant was in possession of the concept that genes and cells are alternative compositions for growing soft tissues in a body. The conversion results illustrate that one skilled in the art could readily understand and apply the dosages of Example 18 to obtain equivalent cell dosage. Validation of the

use of such weight conversion appears to be supported by the fact that such converted dosages are commensurate with those used by workers in the art using bone marrow stem cells to grow an artery, such as that reported in the 2002 Strauer publication (of record and hereinafter “Strauer 2002”).

The Examiner alleged, at page 29 of the Final that the above correspondence of dosages with Strauer 2002 was “pure coincidence” and that Appellant “stumbled upon” a simple method for determining cell numbers. It is clear from such unfounded characterization that the Examiner has paid no deference to the conversion practice used routinely for decades by the medical art. Regarding the alleged “pure coincidence”, attention is directed to the gene and cell dosages of Isner ‘887 at column 11, lines 4-9 and column 7, lines 17-23, respectively. A conversion of the dosages of nucleic acids of Isner ‘887 to corresponding dosages of cells was conducted.²

It is evident from the conversion of nucleic acid dosages to cell dosages that the converted cell dosages fall within the range specified by Isner ‘887. The reasonableness of the conversion has been previously demonstrated regarding a conversion of the dosage of Example 18 in the instant application to the bone marrow stem cell dosages specified by Strauer 2002. Hence, the usefulness of the well-known and established weight conversion has been demonstrated in two instances. Appellant believes this fact constitutes compelling evidence that the Examiner’s criticism of the conversion is unwarranted. The Third Supplemental Declaration of Dr. Richard Heuser (of record and

² Isner ‘887 specified a common dosage of 2000 micrograms for the more preferably and most preferably dosage ranges. Such common dosage was utilized in the conversion calculations. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The 2000 microgram dosage was converted to pg by multiplying by 10^6 equals 2000×10^6 pg. An average weight of 40 pg was used for nucleic acids as consistent with the prior conversion. The conversion was then made by dividing 2000×10^6 by 40 to arrive at a cell dosage of 50×10^6 and falls within the range specified by Isner ‘887.

originally filed in co-pending application Serial No. 10/179,589) and the Second Supplemental Declaration of Dr. Andrew E. Lorincz (of record and originally filed in co-pending application Serial No. 10/179,589) confirm that the use of such well known tool is reasonable in the medical art. Accordingly, Appellant believes that the Examiner's above comments are based upon unsupported speculation and opinion rather than upon evidence.

The Examiner's statement at pages 27 and 28 of the Final that a person of skill in the art "...would never attempt such an extrapolation" is based on the incorrect determination that implanting genes is a technically different process from implanting cells. Such incorrect determination formed the basis for the equally incorrect conclusion that gene therapy and cell therapy have different status in the art and, therefore, cannot be considered as functional equivalents of one another. One need look no further than Isner '887 and Asahara to dispel such erroneous opinion.

The Examiner's statement that "[n]o such extrapolation is taught in the specification," is inept since such extrapolations have been used for decades in the medical arts in regard to cell therapy. That which is well known in the art need not be included in Appellant's specification in order to comply with the enablement requirement of Section 112, first paragraph. See MPEP Section 2164.01.

Appellant believes that the dosage extrapolation and the opinions in regard thereto expressed in the Declarations of Drs. Heuser and Lorincz speak for themselves and confirm the reasonableness of Appellant's conversions. It is of particular note that the extrapolated dosages compare favorably (overlap) with the dosages of global bone marrow cells used by Strauer 2002 for treating myocardial infarction in human patients

and in Isner '887 for a different type of soft tissue growth, thereby confirming the reasonableness of the respective Declarants' opinions.

The Examiner, at page 29 of the Final appears to mistakenly believe that the calculus is "Applicant's formula." The Examiner's challenge in regard to the technical basis underlying the conversions is misdirected. Such challenge should be directed toward the originators of this well known medical tool and workers in the art who used such alleged faulty calculus—not with Appellant's experts who simply confirmed that the calculus was reasonable and found its roots in the medical art because it is notoriously well known that dosages are commonly specified on a weight basis.

The Examiner's *ad hominem* criticism of Appellant's conversion fails to adequately give weight to its evidentiary value. Appellant's evidence establishes as a material fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages because the general practice is to employ multiple doses since stem cell overdosing has not proved to be problematic. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant cell therapy. The Board's attention is again directed to the expert opinions of Drs. Heuser and Lorincz which validate the reasonableness of Appellant's dosage conversions.

At pages 30-33, ¶¶ 37-39 of the Final, the Examiner appears to raise the issue that actual working examples are required to establish enablement. The *armamentaria* underlying the Examiner's rejection is a requirement for actual clinical testing in order for inventions in the medical field to satisfy the enablement requirement of the statute.

The Examiner has cited no authority establishing that prophetic inventions in the medical arts are prohibited by regulation, statute or by the courts. Indeed, it is well established that working examples are not required if the invention is disclosed in a manner that one skilled in the art would be able to practice it. Section 2164.02 of the MPEP states that:

Compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed. An example may be “working” or “prophetic.” A working example is based on work actually performed. A prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved.

One skilled in the art reading the specification at page 46, lines 3-8 would reasonably understand that Appellant disclosed a method comprising seeding, e.g., injecting, appropriate cells (stem cells) or other growth factors to promote growth of blood vessels (arteries) in a damaged portion of a human heart. Furthermore, one reasonably skilled in the art would understand from page 47, line 22 through page 48, line 15 that a patient’s own (autologous) stem cells can be used to grow function specific tissue, such as an artery, *in vivo* through differentiation and morphogenesis. Those skilled in the art understand that “morphogenesis” is the formation and differentiation of tissues and organs and that an artery is an organ. See pages 33 and 48 of the specification.

The Examiner’s statement regarding “writing it down,” absent evidence or sound reasoning, is insufficient to overcome the objective enablement provided by the specification. cf In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Apparently, the Examiner fails to appreciate that the act of “writing down” a “prophetic” example which describes an embodiment based upon predicted results

rather than work actually conducted is sufficient to satisfy a constructive reduction to practice.

The Examiner's statement', at page 30, ¶37 of the Final, that "the instant specification adds no new technical advance beyond that which is taught by Isner et al (Circulation. 1995;912687-2692)," evinces an apparent lack of understanding of the medical arts. The Isner et al. Circulation publication is related solely to gene therapy. Isner '887 confirms Appellant's argument that implanted endothelial progenitor cells (ECs) only attach to capillary walls and are incapable of promoting the growth of an artery as required in the claimed subject matter on appeal. To ignore this is to ignore the best evidence in the record that Appellant's invention is distinct and novel from both the Isner et al. Circulation publication and Isner '887. Appellant has not trivialized the differences between the respective works of Isner and the claimed method. Rather, Appellant has diligently pointed out the differences that exists there between. The exuding of triviality resides solely with the Examiner because the question of whether Appellant has made a new technical advance that is unrelated to the enablement issue on appeal.

Furthermore, it is clear that the Isner et al. Circulation publication did not appreciate that stem cells promote the growth of arteries. It is further clear that Isner '887 failed to appreciate that global bone marrow stem cells promote the growth of arteries. The only support for the Examiner's statement that, "the instant specification does not even begin to work out the procedural differences between the protocol taught by Isner et al Circulation publication, and any method that uses cells instead of cDNA" is the written statement itself. The truth is that no difference in procedural protocol exists

for administering stem cells using a hypodermic needle *vis-a-vis* genes, and this is readily apparent from reading the instant specification at page 21. This fact is confirmed by Isner '887 which teaches muscular injection of genes and cells. The Examiner has failed to identify what protocol is missing from Appellant's specification that would prevent one skilled in the art from practicing the claimed subject matter.

The Examiner, at pages 30 and 31 of the Final, stated that the PTO is forbidden to comment upon the validity of Isner '887, but then curiously proceeded to apparently defend the validity of this patent. Lest there be any misunderstanding, Appellant has never stated that Isner '887 is invalid.

The "obviousness" issue raised at page 33 of the Final has relevance only in the Examiner's mind because Appellant never, intentionally or unintentionally, linked an absence of an art rejection with proof of enablement. In any event, the Examiner has stated, at page 34, that an obviousness rejection would not be proper and thus no issue exists between Appellant and Examiner on this point.

As a final point, the Examiner, at pages 13 and 25 of the Final, refers to the breadth of claims, the amount of direction or guidance, and the presence or absence of working examples as evidence of that undue experimentation would be required to practice the claimed invention, citing In re Wands 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Cir.1988). In Wands, the Court focused on three factors: the state of the prior art, the level of skill in the art, and the amount of direction provided by the specification. The specification (pages 47-48) clearly describes the concept of implanting a patient's own cells (autologous stem cells) to promote differentiation and morphogenesis into an organ, which by disclosure includes an artery. The specification teaches numerous

methods of implantation including intramuscular injection. The Examiner's allegation that the specification fails to address complex problems "that might be encountered" in stem cell therapy is a "red herring," which has not been factually supported on the record. Contemporary prior art wisdom (Isner'887, Asahara) at the time of Appellant's invention demonstrates the conventionality of intramuscular injection of stem cells and genes in treating disease involving ischemic tissue. Contrary to the Examiner's assertion, the post filing work of Strauer 2002 does not describe solving any complex problems associated with implanting bone marrow stem cells. Neither the contemporary nor post filing art disclose any specific problems that had to be addressed and overcome in order to successfully implant cells in a human patient. Thus, the Examiner's determination that the specification is non enabling because it fails to address nonexistent problems is inauthentic.

Appellant believes the instant fact situation is similar to that of Wands because the skill level is high and known administration techniques and known materials are utilized in the practice of the invention. In addition to such factual parallelism, Appellant provided expert objective evidence in Paragraph 7 of the Fourth Supplemental Declarations of Drs. Heuser and Lorincz. These medical experts read portions of the specification setting forth the generic growth factor invention and claimed and non-claimed species of such generic invention and determined that one skilled in the medical arts, armed with the guidance and direction in the relevant specification disclosures, would be enabled to practice the methods defined in the claims on appeal and to predictably anticipate the results defined therein without need for resorting to undue experimentation. When the guidance and direction provided by Appellant's specification

disclosure, the level of knowledge and the content of the prior at the time of the invention, such as that of Isner '887, Asahara and Nabel, as established in the record and Appellant's declaration evidence are interpreted in a reasonable manner, an analysis considering the Wands factors compels a conclusion that undue experimentation would not be required to practice the invention called for in the appealed claims.

In summary, Appellant believes that the Examiner failed to provide sufficient objective evidence or reasoning to support a determination of lack of enablement under current law when considered *vis-à-vis* the evidence of enablement provided by Appellant's specification. Thus, the Examiner has failed to establish a *prima facie* case of lack of enablement, and this rejection should be withdrawn.

Assuming *arguendo*, that the Examiner somehow met the burden of establishing a *prima facie* case of lack of enablement, Appellant believes that any such case has been fully rebutted by the submission of the multiple Declarations of experts in the medical field—Drs. Meger, Lorincz, and Heuser (The Declaration of Dr. Meger; the Declarations, Supplemental Declarations, Second Supplemental Declarations, Third Supplemental Declarations, Fourth Supplemental Declarations, and the Declarations referred to above that were originally filed in co-pending application Serial No. 10/179,589, of Drs. Heuser and Lorincz, all of record). The conclusions set forth in these multiple Declarations establish material facts relating to a determination of description and enablement regarding the subject matter of the claims on appeal. These highly skilled medical experts read and relied solely upon identified, relevant portions of the specification, including generic, elected, and non-elected species portions, and reached independent determinations that one skilled in the medical art, armed with the

knowledge presented in Appellant's disclosures, would be enabled to practice the claimed method and to predictably anticipate the results defined therein without need for resorting to undue experimentation.

The Examiner's failure at page 43, ¶41 to consider the merits of the above-mentioned multiple Declarations, and thus erroneously according "no weight" to such evidence, constitutes reversible error. The Examiner's failure to critically analyze and accord weight to Appellant's declaration evidence constitutes error as a matter of law. In re Alton, supra. It is trite law that the Examiner must consider the probative value of such evidence *vis-à-vis* any asserted *prima facie* case. See In re Oetiker, at 1445, 24 USPQ 2d at 1444. In re Keller, 642 F.2d 413, 208 USPQ 871, (CCPA 1981). The Examiner, not being a skilled person in the medical art, must give weight to these expert opinions rather than rely solely upon his own opinion. See In re Neave, 370 F.2d 961, 152 USPQ 274, (CCPA 1967).

For the above reasons, Appellant submits that the rejection for lack of enablement under 35 U.S.C. §112, second paragraph, is contrary to current law, and perforce, should be withdrawn.

Provisional Rejection Nonstatutory – Obviousness-Type Double Patenting

Claims 403-405 were provisionally finally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 163, and 170-173 of co-pending application Serial No. 10/179,589. Appellant again notes such rejection

and stands ready to submit an appropriate Terminal Disclaimer upon an indication of allowable subject matter related to such claims.

Rejection under 35 U.S.C. §101 – Double Patenting

Claims 403 and 407-412 were provisionally finally rejected under 35 U.S.C. §101 as claiming the same invention as that of claims 161-164 and 172-174 of co-pending Application Serial No. 10/179,589. Appellant notes that claim 403 was also rejected upon obvious-type double patenting grounds and thus the respective rejections appear on their face to be inconsistent.

It is pointed out that the claims in the instant application require the preliminary step of forming a bud in the body of the patient which then grows into an artery, while the claims co-pending application Serial No. 10/179,589 have no such requirement. Hence the claims presented in the respective applications are not drawn to identical subject matter. Hence, claims 403 and 407-412 are not identical with claims 161-164 and 172-174,

It is apparent from the above paragraphs that the respective claims are not drawn to the same invention and that the Examiner committed error. Accordingly, it is submitted by Appellant that the provisional final rejection under 35 U.S.C. §101 should be reversed by the Board.

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CONCLUSION AND RELIEF SOUGHT

In view of the foregoing, Appellant urges the Board to reverse the outstanding rejections of claims 403-405 and 407-412 and respectfully requests that the instant application be passed to issue.

Respectfully submitted,

Dated: 10/06/08



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CLAIMS APPENDIX

Claims 403-405 and 407-412 are pending in the application, are under final rejection, are being appealed, and are listed below.

LISTING OF CLAIMS

- Claim 403 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:
- (a) locally injecting stem cells into said body at said selected site;
 - (b) forming a bud at said selected site; and
 - (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.
- Claim 404 The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
- Claim 405 The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

- Claim 407 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.
- Claim 408 The method of claim 407, wherein said bone marrow is from said patient.
- Claim 409 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.
- Claim 410 The method of claim 409, wherein said blood is from said patient.
- Claim 411 The method of claim 403 further comprising determining blood flow through said desired artery.
- Claim 412 The method of claim 403 further comprising observing said desired artery.

EVIDENCE APPENDIX

1. Isner U.S. Patent No. 5,980,887 cited by Appellant as Exhibit A in the Response filed November 28, 2007.
2. Asahara, et al., 1997 Science article entitled, "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis" cited by Appellant as Exhibit A in Appeal Brief filed herewith.
3. Declaration of Dr. Richard Heuser filed November 22, 2004.
4. Supplemental Declaration of Dr. Heuser filed June 20, 2005.
5. Second Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit C in the Response filed June 26, 2006.
6. Third Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit C in the Response filed April 30, 2007.
7. Fourth Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit D in the Response filed November 28, 2007.
8. Third Supplemental Declaration of Dr. Heuser (originally filed in co-pending application Serial No. 10/179,589) and cited by Appellant as Exhibit B in the Letter filed May 25, 2007.
9. Declaration of Dr. Andrew E. Lorincz cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.
10. Supplemental Declaration of Dr. Andrew E. Lorincz filed November 15, 2004.
11. Second Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit D in the Response filed June 26, 2006.
12. Third Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit B in the Response filed April 30, 2007.
13. Fourth Supplemental Declaration of Dr. Andrew E. Lorincz cited by Appellant as Exhibit E in the Response filed November 28, 2007.
14. Second Supplemental Declaration of Dr. Andrew E. Lorincz (originally filed in co-pending application Serial No. 09/794,456) and cited by Appellant as Exhibit A in the Letter filed May 25, 2007.

15. Office Action issued on February 22, 2006 in co-pending application Serial No. 09/794,456 by Examiner Kemmerer, page 6, lines 1-8, cited by Appellant as Exhibit B in Appellant's instant Appeal Brief filed herewith.
16. Nabel U.S. Patent No. 5,328,470 cited by Appellant as Reference AD in the Information Disclosure Statement filed February 15, 2001.
17. Kornowski U.S. Patent No. 7,097,832 cited by Appellant as Exhibit B in the Response filed November 28, 2007.
18. American Heart Association 2004 article titled, "Endothelial Progenitor Cells: More than an Inflammatory Response?" cited by Appellant as Exhibit C in the Response filed November 28, 2007.
19. Declaration of Dr. G. Robert Meger cited by Appellant as an Exhibit in the Amendment filed February 15, 2001.
20. Strauer et al. 2002 publication in Circulation entitled, "Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans" cited by Appellant as Reference ABQ in the Third Supplemental Information Disclosure Statement filed May 27, 2003.

RELATED PROCEEDINGS APPENDIX

1. Application Serial No. 10/179,589 filed June 25, 2002, Appellant's Appeal Brief filed on August 28, 2008.
2. Application Serial No. 09/794,456 filed February 27, 2001, Notice of Appeal mailed on September 30, 2008.
3. Application Serial No. 09/836,750 filed April 27, 2001, Appellant's Reply Brief filed on March 18, 2008. Request for Continued Examination filed on June 6, 2008.

EXHIBIT A

**Asahara, et al. 1997 Science article entitled,
“Isolation of Putative Progenitor
Endothelial Cells for Angiogenesis”**

Isolation of Putative Progenitor Endothelial Cells for Angiogenesis

Takayuki Asahara, Toyoaki Murohara, Alison Sullivan,
Marcy Silver, Rien van der Zee, Tong Li,
Bernhard Witzentichler, Gina Schatteman, Jeffrey M. Isner*

Putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and for delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis.

Postnatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to the formation of embryonic blood vessels from EC progenitors, or angioblasts (2).

Vasculogenesis begins as a cluster formation, or blood island, comprising angioblasts at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this spatial association, angioblasts and HSCs share certain antigenic determinants, including Flk-1, Tie-2, and CD34. Conceivably, then, these progenitor cells may derive from a common precursor (3, 4).

The demonstration that HSCs from peripheral blood can provide sustained hematopoietic recovery is inferential evidence for circulating stem cells (5). Here, we have investigated the hypothesis that peripheral blood contains cells that can differentiate into ECs (6). We exploited two antigens that are shared by angioblasts and HSCs to isolate putative angioblasts from the leukocyte fraction of peripheral blood. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (7). It is also expressed by many including most activated ECs in the adult (8). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (9), is also expressed by both early HSCs and ECs but ceases to be expressed during hematopoietic differentiation (10, 11).

CD34-positive mononuclear blood cells (MB^{CD34+}) were isolated from human peripheral blood by means of magnetic beads

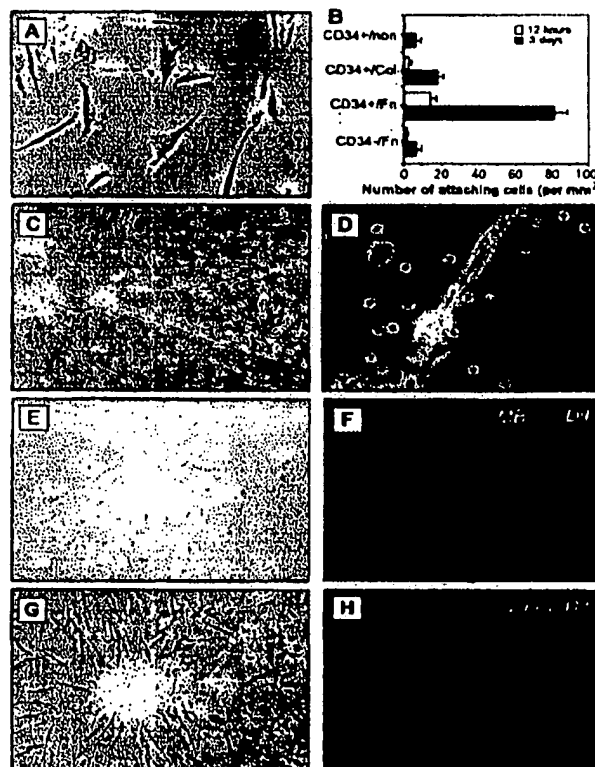
coated with antibody to CD34 (Dyna, Lake Success) (12). Fluorescence-activated cell sorting (FACS) analysis (13) indicated that $15.7 \pm 3.3\%$ of selected cells compared with $<0.1\%$ of the remaining cells expressed CD34. CD34-depleted cells (MB^{CD34-}) were used as controls. An antibody to Flk-1 was used for magnetic bead selection of Flk-1-positive mononuclear

blood cells (MB^{Flk1+}); among MB^{Flk1+} cells, $20.0 \pm 3.3\%$ were Flk-1 positive.

The MB^{CD34+} and MB^{CD34-} cells were plated separately (14) on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of 1×10^5 cells/mm², a limited number of MB^{CD34+} attached, became spindle shaped, and proliferated for 4 weeks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (Fig. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (probability $P < 0.05$, by analysis of variance) (Fig. 1B). Attached cells were observed only sporadically among MB^{CD34-} cultures, including cells followed for up to 4 weeks on fibronectin-coated plates.

To confirm that the spindle-shaped cells were derived from CD34-positive cells, we labeled MB^{CD34+} cells with the fluorescent dye Dil and coplanted them with unlabeled MB^{CD34-} cells on fibronectin at an overall density of 5×10^5 cells/mm²; the ratio of the two cell types was identical to that of the original mononuclear cell population ($1\% MB^{CD34+}$, $99\% MB^{CD34-}$). After 7 days, Dil-labeled cells derived from the MB^{CD34+} culture, which initially account-

Fig. 1. Attachment, cluster formation, and capillary network development by progenitor ECs in vitro. (A) Spindle-shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} (50 cells/mm²) on fibronectin in standard medium (14). (B) Number of AT^{CD34+} cells 12 hours and 3 days after culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/Col), or fibronectin (CD34+/Fn), and MB^{CD34-} on fibronectin (CD34-/Fn). Network formation (C) and cord-like structures (D) were observed 48 hours after plating coculture of MB^{CD34+} , labeled with Dil, with unlabeled MB^{CD34-} cells (ratio of 1:100) on fibronectin. At 12 hours after coculture, MB^{CD34+} -derived cells had formed multiple clusters (E and F). After 5 days, uptake of acLDL-Dil was detected in AT^{CD34+} cells at the periphery but not the center of the cluster (G and H).



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ed for only 1% of the blood cells, accounted for $60.3 \pm 4.7\%$ of total attaching cells as analyzed by FACS. Coincubation with MB^{CD34+} cells increased the proliferation rate to more than 10 times that of MB^{CD34+} plated alone. Cocultures of MB^{CD34+} and MB^{CD34+} cells also showed enhanced MB^{CD34+} differentiation, including the formation of cellular networks and tube-like structures on fibronectin-coated plates (Fig. 1, C and D). These structures consisted principally of Dil-labeled MB^{CD34+} -derived cells (Fig. 1D). Furthermore, within 12 hours of coculture, multiple clusters had formed (Fig. 1E) that contained mostly MB^{CD34+} -derived cells (Fig. 1F). These clusters comprised round cells centrally and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to ECs and vascular structures *in vitro* (3). AT^{CD34+} cells at the cluster periphery took up Dil-labeled acetylated low density lipoprotein (acLDL), whereas the round cells did not (Fig. 1, G and H); the latter detached from the cluster several days later. The MB^{Flk-1+} cells behaved similarly.

To evaluate whether MB^{CD34+} cells progressed to an EC-like phenotype, we assayed them for the expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} cells, AT^{CD34+} cells cultured on fibronectin for 7 days, and human umbilical vein endothelial cells (HUVECs) were incubated with fluorescent-labeled antibodies and analyzed by FACS (Fig. 2). Leukocyte common antigen CD45 was identified on 94.1% of freshly

isolated cells but disappeared after 7 days of culture (Fig. 2). In freshly isolated MB^{CD34+} cells, $15.7 \pm 3.3\%$ were $CD34+$, $27.6 \pm 4.3\%$ were $Flk-1+$, and $10.8 \pm 0.9\%$ were $CD34+Flk-1+$. Expression of $CD34$, $CD31$, $Flk-1$, $Tie-2$, and E selectin—all markers of the EC lineage (11, 15)—was greater in AT^{CD34+} cells after 7 days of culture than in freshly isolated MB^{CD34+} cells.

Additional analyses (16) of AT^{CD34+} cells after 7 days of culture showed limited ($6.0 \pm 2.4\%$ cells) expression of CD68, a marker of the monocyte-macrophage lineage; positive immunostaining for factor VIII, ulex europaeus agglutinin-1 (UEA-1), CD31, endothelial constitutive nitric oxide synthase (eNOS), and E selectin; and more than 80% uptake of Dil-labeled acLDL.

To confirm an EC-like phenotype of AT^{CD34+} cells, we documented expression of eNOS, $Flk-1/KDR$ ($Flk-1$ is also known as $VEGFR-2$ in mouse, and KDR is the human homolog of $VEGFR-2$), and $CD31$ mRNA at 7, 14, and 21 days by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Evidence for eNOS and $Flk-1/KDR$ in AT^{CD34+} cells was also demonstrated in a functional assay. Nitric oxide was produced in the cells in response to the EC-dependent agonist acetylcholine (ACh) and the EC-specific mitogen VEGF (Fig. 3B); the latter response also confirms that the cells express a functional $Flk-1$ receptor (17).

To determine if MB^{CD34+} cells contribute to angiogenesis *in vivo*, we used mouse and rabbit models of hindlimb ischemia. For administration of human MB^{CD34+} cells, C57BL/6J \times 129/SV background athymic

nude mice were used to avoid potential graft-versus-host complications. Two days after creating unilateral hindlimb ischemia by excising one femoral artery, we injected mice with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34+} cells into the tail vein. Histologic examination 1 to 6 weeks later revealed numerous (Fig. 4A) including proliferative (Fig. 4, C and D) Dil-labeled cells in the neovascularized ischemic hindlimb. Nearly all labeled cells appeared integrated into capillary vessel walls. In MB^{CD34+} -injected mice, $13.4 \pm 5.7\%$ of all $CD31$ -positive capillaries contained Dil-labeled cells, compared with $1.6 \pm 0.8\%$ in MB^{CD34+} -injected mice (18). By 6 weeks, Dil-labeled cells were clearly arranged into capillaries among preserved muscle structures (Fig. 4, I and J).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34+} -injected mice. Dil-labeled cells consistently colocalized with cells immunostained for $CD31$ (Fig. 4, B, F, and J), $Tie-2$ (Fig. 4G), and UEA-1 lectin (16). In contrast, in hindlimb sections from mice injected with MB^{CD34+} , Dil-labeled cells were typically found in stroma near capillaries, but they did not form part of the vessel wall nor did they colocalize with cells that stained with antibodies to either UEA-1 or $CD31$ (Fig. 4, K and L).

In a second set of mouse experiments, 1×10^4 MB^{Flk-1+} cells were isolated from

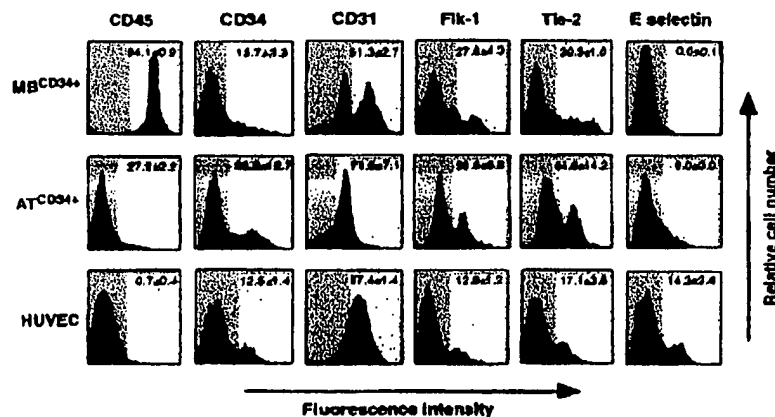


Fig. 2. FACS analysis of freshly isolated MB^{CD34+} and AT^{CD34+} cells after 7 days in culture, and HUVECs. Cells were labeled with fluorescent antibodies to CD45 (DAKO, Carpinteria); CD34, CD31 (Bioss); $Flk-1$, $Tie-2$ (Santa Cruz); and E selectin (DAKO). Similar results were obtained in three or more experiments. The shaded area of each box denotes negative antigen gate, and the white area denotes positive gate. Numbers are the mean \pm SEM percentage of cells for all experiments determined by comparison with corresponding negative control labeling.

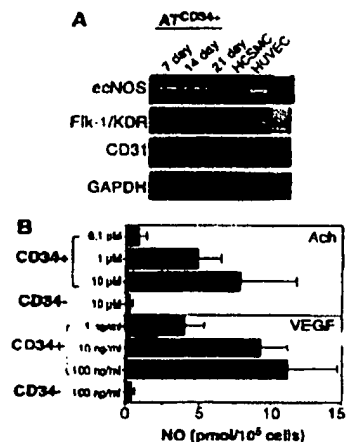


Fig. 3. Progenitor ECs express eNOS, $Flk-1/KDR$, and $CD31$ mRNA and release NO. (A) Complementary DNA (from 10^5 cells) was amplified by PCR (40 cycles) with paired primers (23). (B) NO release from AT^{CD34+} and AT^{CD34+} cells cultured in six-well plates was measured as described (24). NO production was measured in a well with incremental doses of VEGF and ACh. HUVECs and bovine aortic ECs were used as positive controls, and human coronary smooth muscle cells (HSCMCs) as negative control. The values are means \pm SEM of 10 measurements for each group.

whole blood of 10 transgenic mice constitutively overexpressing β -galactosidase (β -Gal) (all mice were Flk-1^{+/+}). MB^{Flk-1+} or MB^{Flk-1-} cells were injected into nontransgenic mice of the same genetic background that had hindlimb ischemia of 2 days duration. Immunostaining of ischemic tissue, harvested 4 weeks after injection, for β -Gal demonstrated incorporation of cells expressing β -Gal in capillaries and small ar-

teries (Fig. 4M); these cells were identified as ECs by staining with antibody to CD31 (anti-CD31) and BS-1 lectin.

In vivo incorporation of autologous MB^{CD34+} cells into foci of neovascularization was also tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} cells were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand White rabbits immediately

before surgical induction of unilateral hindlimb ischemia (19). Immediately after surgery, freshly isolated autologous Dil-labeled MB^{CD34+} were reinjected into the ear vein of the same rabbit. Histologic examination of the ischemic limbs 4 weeks later revealed that Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb (Fig. 4, N and O) and were incorporated into $9.7 \pm 4.5\%$ of the capillaries that

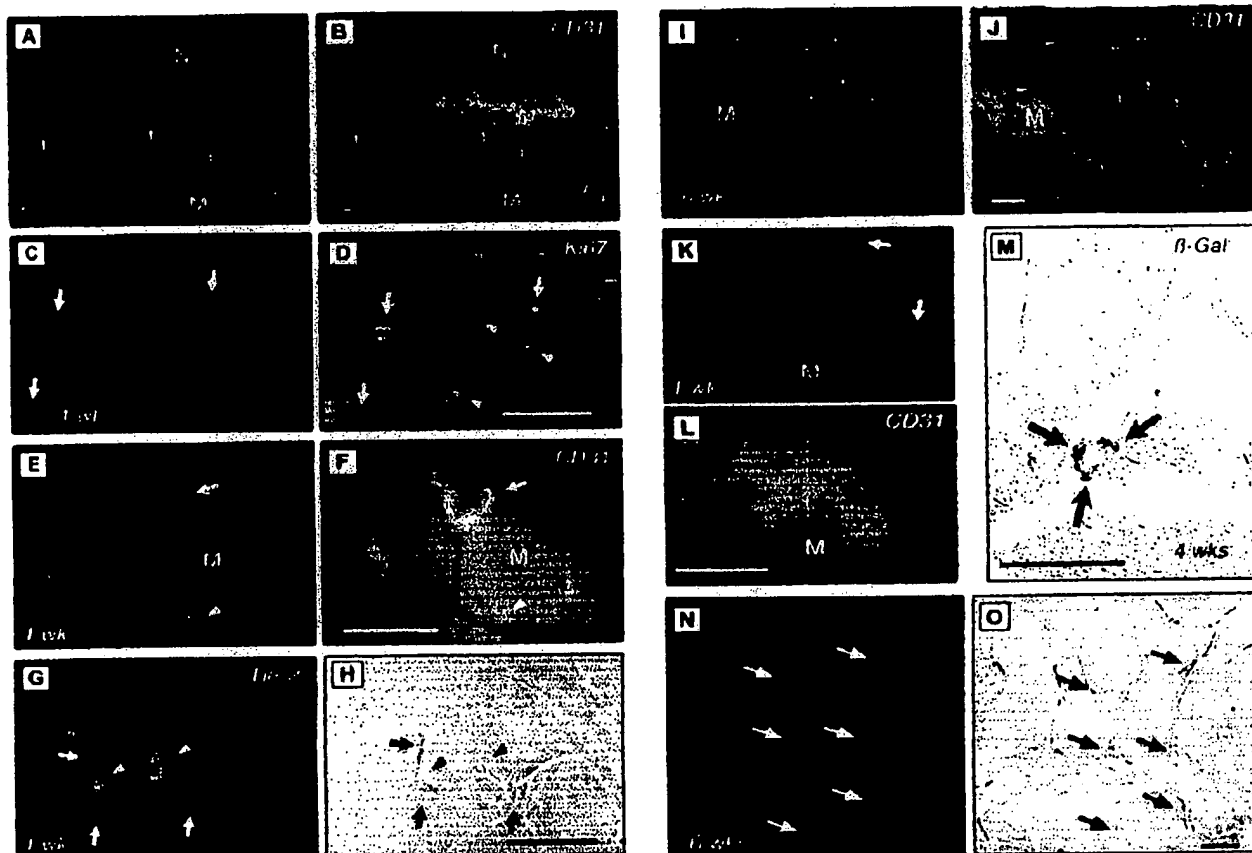


Fig. 4. Heterologous (panels A to L), homologous (M), or autologous (panels N and O) EC progenitors incorporate into sites of angiogenesis in vivo. (A and B) Dil-labeled MB^{CD34+} (red, arrows) between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are labeled with CD31 (green, arrows). Note a preexisting artery (A), identified as CD31-positive, but Dil-negative. (C and D) Evidence of proliferative activity among several Dil-labeled MB^{CD34+}-derived cells (red, arrows), indicated by colmunostaining for antibody to Ki67 (Vector Lab, Burlingame, California) (green). Proliferative activity is also seen among Dil-negative, Ki67-positive capillary ECs (arrowheads); both cell types contribute to neovascularization. (E) Dil (red) and CD31 (green) in capillary ECs (arrows in E and F) between skeletal myocytes, photographed through a double filter 1 week after Dil-labeled MB^{CD34+} injection. (F) A single green filter shows CD31 (green) expression in Dil-labeled capillary ECs integrated into the capillary with native (Dil-negative, CD31-positive) ECs (arrowheads in E and F). (G) Immunostaining 1 week after MB^{CD34+} injection showing capillaries comprising Dil-labeled MB^{CD34+}-derived cells expressing Tie-2 receptor (green). Several MB^{CD34+}-derived cells (arrows) Tie-2 positive and integrated with some Tie-2-positive

host capillary cells (arrowheads) identified by the absence of red fluorescence. (H) Phase-contrast photomicrograph of the same section shown in (G) indicates the corresponding Dil-labeled (arrows) and -unlabeled (arrowheads) capillary ECs. (I and J) Six weeks after administration, MB^{CD34+}-derived cells (red, arrows) colabel for CD31 in capillaries between preserved skeletal myocytes (M). (K and L) One week after injection of MB^{CD34+}, isolated MB^{CD34+}-derived cells (red, arrows) are observed between myocytes but do not express CD31. (M) Immunostaining of β -Gal in a tissue section harvested from ischemic muscle of C57BL/6J, 129/SV mice 4 weeks after the administration of MB^{Flk-1+} isolated from transgenic mice constitutively expressing β -Gal (arrows) were incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and BS-1 lectin (16). (N and O) Section of muscle harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous MB^{CD34+} cells. Red fluorescence in (N) indicates localization of MB^{CD34+}-derived cells in capillaries seen (arrows) in the phase-contrast photomicrograph in (O). Each scale bar is 50 μ m.

consistently expressed CD31 and reacted with BS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti-Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk-1+} cells may contribute to neovascularization in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2, 3). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (20) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (21). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For anti-neoplastic therapies, MB^{CD34+} cells could be transduced with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (22).

REFERENCES AND NOTES

1. J. Folkman and Y. Shing, *J. Biol. Chem.* **267**, 10931 (1992); W. Schaper, M. D. Brahander, P. Lawl, *Circ. Res.* **28**, 671 (1971); W. Risau, *FASEB J.* **9**, 926 (1995).
2. W. Risau et al., *Development* **102**, 471 (1988); L. Perdenaud, C. Altman, P. Khos, F. Diesterton-Llewre, *ibid.* **105**, 473 (1989).
3. I. Flamme and W. Risau, *ibid.* **116**, 435 (1992).
4. W. His, *Abhandl. K.S. Ges. Wiss. Math.-Phys.* **22**, 171 (1900); M. Weiss and S. H. Orkin, *J. Clin. Invest.* **97**, 591 (1996).
5. W. Brugger, S. Helmelt, R. J. Berenson, R. Mertelsmann, L. Kanz, *N. Engl. J. Med.* **333**, 283 (1995).
6. M. Wu et al., *J. Vasc. Surg.* **21**, 862 (1995); Q. Shi et al., *ibid.* **20**, 546 (1994); Y. Nishikawa, Y. Tomizawa, Y. Yamano, A. Matsunaga, *Nature Med.* **2**, 90 (1996).
7. C. I. Chin et al., *J. Immunol.* **133**, 157 (1984); F. Katz, R. W. Tindle, D. H. Sutherland, M. D. Greaves, *Leukemia Res.* **9**, 191 (1985); R. G. Andrews, J. W. Singer, I. D. Bernstein, *Blood* **67**, 842 (1986).
8. J. Fria et al., *Blood* **75**, 2417 (1990); D. Soligo et al., *Leukemia (Lusingsloek)* **5**, 1026 (1991); A. Ito, S. Nomura, S. Hirota, I. Suda, Y. Kitamura, *Lab. Invest.* **72**, 532 (1995).
9. C. deVries et al., *Science* **255**, 989 (1992); B. I. Terman et al., *Biochem. Biophys. Res. Commun.* **187**, 1579 (1992); F. Shalaby et al., *Nature* **376**, 62 (1995).
10. W. Matthews et al., *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9026 (1991).
11. B. Milbauer et al., *Cell* **72**, 835 (1993); T. P. Yamaguchi, D. J. Dumont, R. A. Conlon, M. L. Breitman, J. Rossant, *Development* **118**, 449 (1993).
12. Single donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
13. MB^{CD34+} , MB^{CD34-} , and MB^{Flk-1+} cells ($>1 \times 10^6$ of each) were analyzed with anti-CD34 (Bioss, Kennebunkport, ME) and anti-Flk-1 (Santa Cruz Biotechnologies, Santa Cruz, CA).
14. The medium for all cell culture experiments was M-199 with 20% fetal bovine serum and bovine brain extract (Cibacel, San Diego).
15. P. J. Newman et al., *Science* **247**, 1219 (1990); T. N. Satoh et al., *Nature* **376**, 70 (1995); H. Schnurich and W. Risau, *Development* **119**, 957 (1993); M. P. Bevilacqua, *Arterioscler. Thromb.* **11**, 767 (1993).
16. T. Asashara et al., data not shown.
17. H. van der Zee et al., *Circulation*, in press.
18. The mean percent of DiI-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields ($\times 400$).
19. New Zealand White rabbits (3.8 to 4.2 kg, $n = 4$, Pine Acres Rabbits, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised [S. Takeshita et al., *J. Clin. Invest.* **93**, 662 (1994); L. O. Fu et al., *Circulation* **68**, 208 (1993); R. Baffour et al., *J. Vasc. Surg.* **16**, 181 (1992)].
20. J. M. Isner et al., *Lancet* **348**, 370 (1996).
21. M. R. Tschudi et al., *J. Clin. Invest.* **98**, 899 (1996).
22. Y. Sato et al., *Exp. Cell Res.* **204**, 223 (1993); M. S. Pepper, N. Ferrara, L. Orci, R. Montesano, *Biochem. Biophys. Res. Commun.* **181**, 902 (1991); D. N. Senger et al., *Am. J. Pathol.* **149**, 293 (1996).
23. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for eNOS, AAG ACA TTT TCG GGC TCA CGC TGC GCA CCG/ TGG GGT AGC CAC TTT AGT AGT TGT CCT AAC [548-bp pairs (bp) PCR product]; for Flk-1 (KDR), CAA GAA AGT CCG GAG AGG AG/ATG ACG ATG GAC AAG TAG CC (819-bp PCR product); for CD31, GCT G1 T GGT GGA AGG AGT GC/GAA GTT GGC TGG AGG TGC TC (845-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/ CAT GTG GGC CAT GAG GTC CAC CAC (983-bp PCR product).
24. NO release was measured with a NO-specific polarographic electrode connected to a NO meter (iso-NO, World Precision Instruments, Sarasota, FL). ATC^{200+} or ATC^{204} cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.
25. Supported by grants from NIH National Heart, Lung, and Blood Institute numbers 02824, 53354, and 57516, the American Heart Association, the E. L. Wiegand Foundation, and in part by the Uehara Memorial Foundation (T.M.).

4 October 1996; accepted 14 January 1997

Somatic Frameshift Mutations in the BAX Gene in Colon Cancers of the Microsatellite Mutator Phenotype

Nicholas Rampino, Hiroyuki Yamamoto, Yuriy Ionov, Yan Li, Hisako Sawai, John C. Reed, Manuel Perucho*

Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP⁺ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [(G)₈] within BAX, a gene that promotes apoptosis. These mutations were absent in MMP⁻ tumors and were significantly less frequent in (G)_n repeats from other genes. Frameshift mutations were present in both BAX alleles in some MMP⁺ colon tumor cell lines and in primary tumors. These results suggest that inactivating BAX mutations are selected for during the progression of colorectal MMP⁺ tumors and that the wild-type BAX gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (1-3). The fixation of these slip-py-induced replication errors as mutations (4) is associated with defects in DNA mismatch repair (5). Colorectal MMP⁺ tumors frequently contain frameshift mutations in the type II transforming growth factor- β (TGF- β) receptor gene (6) but are usually wild type for the p53 tumor suppressor gene (1, 7). In addition to its central role in cell growth arrest (8), p53 also plays a role in apoptosis in response to DNA

damage (9). The p53 protein transactivates BAX (10), a member of the BCL2 gene family (11) that promotes apoptosis (12).

The human BAX gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (12). To determine whether this sequence is a mutational target in MMP⁺ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the (G)₈ tract from various MMP⁺ tumor cell lines. This analysis revealed hard shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a BAX fragment with mutant (G)₉ and (G)₇ tracts (Fig. 1A, P9 and P7).

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EXHIBIT B

**February 22, 2006 Office Action
Issued in co-pending application
Serial No. 09/794,456, page 6, lines 1-8**

Art Unit: 1646

products, not method steps. The issue here is not whether or not workers in this technology already knew the features of the cells recited in the claims; rather, the issue is that the instant specification did not set forth contemplation of a method step wherein cells were administered intravenously, intraluminally, or via angioplasty. As discussed in the previous paragraph, the instant specification did not set forth contemplation of such method steps. The claims are being examined to the extent they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. Furthermore, MPEP § 2163.02 reads:

"An Applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. See Lockwood v. American Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention."

In the instant case, none of these criteria have been met. There was no reduction to practice, and the specification only refers to method steps involving proteins, genes and "genetic material," *but not cells*, as being useful in intravenous, intraluminal and angioplasty delivery. Therefore, the rejection is maintained.

35 U.S.C. § 112, First Paragraph – Enablement

COPIES OF ITEMS 1-20

Cited in

EVIDENCE APPENDIX

EVIDENCE APPENDIX

ITEM NO. 1

Isner U.S. Patent No. 5,980,887



USC 8-87A

United States Patent [19]**Isner et al.**[11] **Patent Number:** **5,980,887**[45] **Date of Patent:** **Nov. 9, 1999**[54] **METHODS FOR ENHANCING
ANGIOGENESIS WITH ENDOTHELIAL
PROGENITOR CELLS**[75] **Inventors:** **Jeffrey M. Isner, Weston; Takayuki
Asahara, Arlington, both of Mass.**[73] **Assignee:** **St. Elizabeth's Medical Center of
Boston, Boston, Mass.**[21] **Appl. No.:** **08/744,882**[22] **Filed:** **Nov. 8, 1996**[51] **Int. Cl.⁶** **A61K 35/12; A61K 48/00;
A61K 38/18; A61K 38/19**[52] **U.S. Cl.** **424/93.7; 424/85.1; 424/85.2;
514/8; 514/44**[58] **Field of Search** **424/93.7, 85.4,
424/85.2; 435/325, 375; 514/2, 8, 44; 530/351;
53/23.5**[56] **References Cited****U.S. PATENT DOCUMENTS**5,612,211 3/1997 Wilson et al. .
5,652,225 7/1997 Isner .**FOREIGN PATENT DOCUMENTS**WO 89/03875 5/1989 WIPO .
WO 92/07573 5/1992 WIPO .
WO 93/13807 7/1993 WIPO .
WO 96/06933 3/1996 WIPO .
WO 97/12519 4/1997 WIPO .
WO 97/30083 8/1997 WIPO .**OTHER PUBLICATIONS**Isner, et al., *The Lance*, 348:370-374 (1996) (previously
submitted).
XP-002059362-Asahara, et al. "In Vitro Evidence for
Endothelial Cell Precursors in Peripheral Blood". *Circula-
tion* 84(8) p. 1-102 (1996).
XP-002059358-Asahara, et al. "Blood Cell Drived Endot-
helial Cell Precursor Can Participate . . ." *Circulation* 94(8)
p. 1237 (1996) Dec. 7, 1998.
Asahara, et al., *Science* 275: 964-967 (1997) (previously
submitted).Takeshita, et al., *Lab. Inves.*, 75:4, 487-501(1996) (previ-
ously submitted).Schnurch et al. *Development* 119: 957-968 (1993).Yamaguchi et al. *Development* 118: 489-498 (1993).Fennie et al. *Blood* 86: 4454-4467 (1995).Baumgartner et al. *Circulation* 97: 1114-1123 (1998)
Abstract Only.Tsurumi et al. *Circulation* 96 (9 Suppl)II: 382-388 (1997)
Abstract Only.Asahara et al. *Science* 275: 964-967 (1997).Asahara et al. *Circulation* 94(8 Suppl) 1237 (1996).Asahara et al. *Circulation* 92(Suppl II) II-365-371 (1995).Bik Falvi et al. *Leukemia* 8:523-529 (1994).Noishiki et al. *Nature Medicine* 2:90-92 (1996).Shi et al. *J Vas Surg* 20: 546-557 (1994).Folkman and Shing, *J. Biol. Chem.* 267 (16), 10931-109034
(1992).Baffour, et al., *J Vasc Surg*, 16:181-91 (1992).Takeshita, et al., *Circulation*, 90:228-234 (1994).Takeshita, et al., *Laboratory Investigation*, 75:487-502
(1996).Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992).Pu, et al., *J Surg Res*, 54:575-83 (1993).Isner, et al., *Lancet*, 348:370 (1996).Takeshita, et al., *J Clin Invest*, 93:662-70 (1994).**Primary Examiner**—Christina Y. Chan**Assistant Examiner**—Phillip Gambel**Attorney, Agent, or Firm**—David G. Conlin; David S.
Resnick; Dike, Bronstein, Roberts & Cushman, LLP

[57]

ABSTRACTIn accordance with the present invention, EC progenitors
can be used in a method for regulating angiogenesis, i.e.,
enhancing or inhibiting blood vessel formation, in a selected
patient and in some preferred embodiments for targetting
specific locations. For example, the EC progenitors can be
used to enhance angiogenesis or to deliver an angiogenesis
modulator, e.g. anti- or pro-angiogenic agents, respectively
to sites of pathologic or utilitarian angiogenesis.
Additionally, in another embodiment, EC progenitors can be
used to induce reendothelialization of an injured blood
vessel, and thus reduce restenosis by indirectly inhibiting
smooth muscle cell proliferation.**11 Claims, 7 Drawing Sheets**

FIG. 1A



FIG. 1B



FIG. 1C

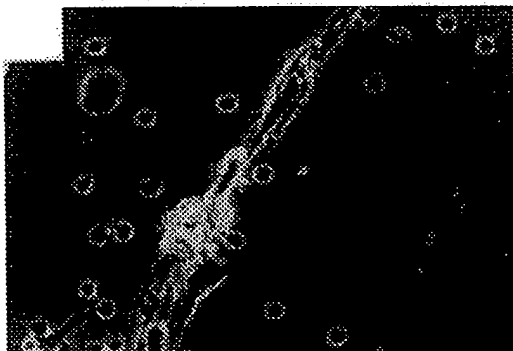


FIG. 1D

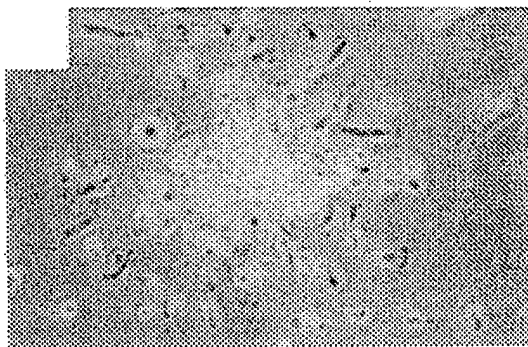


FIG. 1E



FIG. 1F

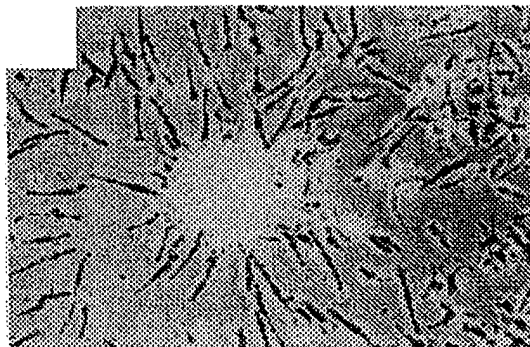


FIG. 1G



FIG. 2

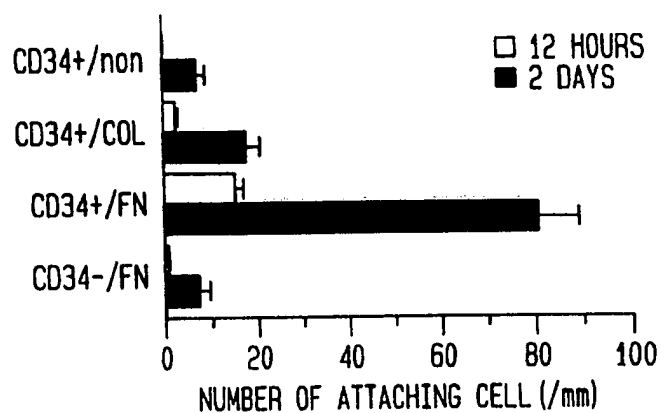


FIG. 5

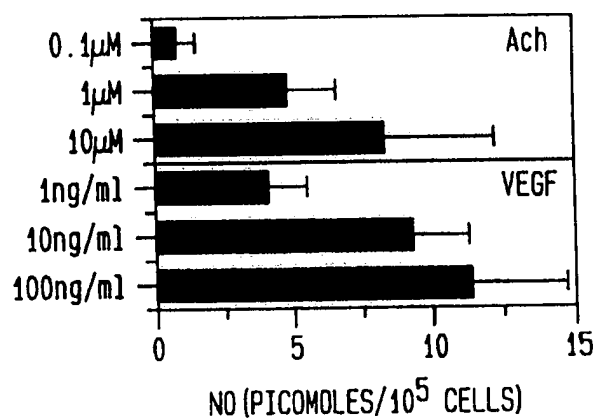


FIG. 7

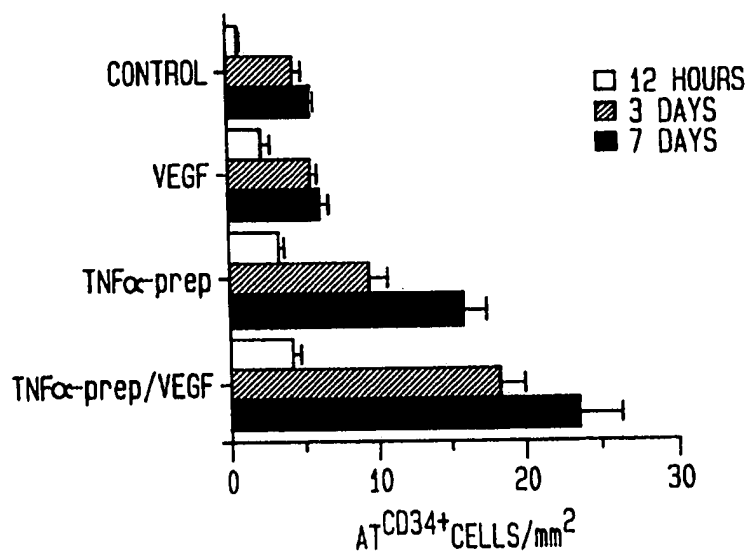


FIG. 3

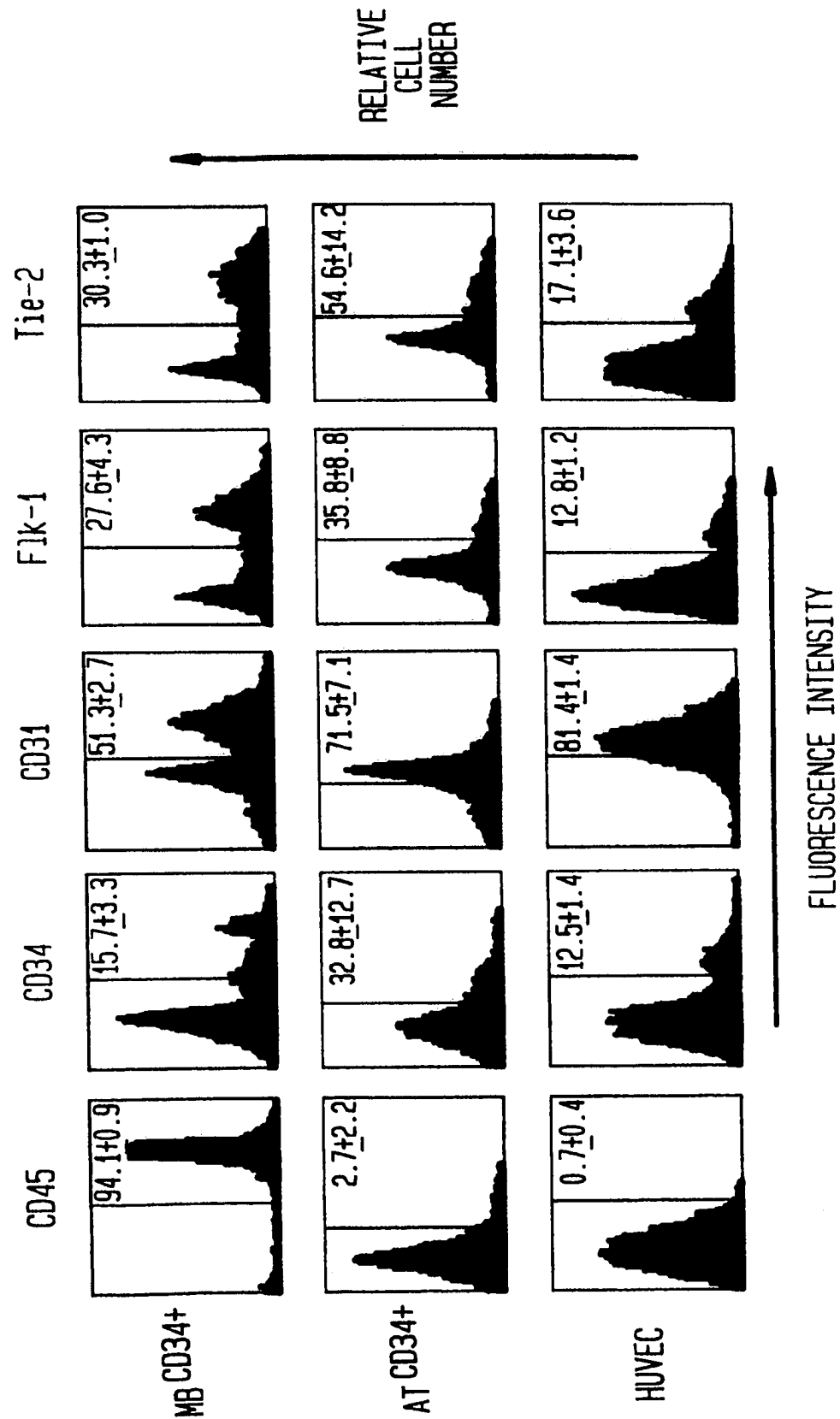


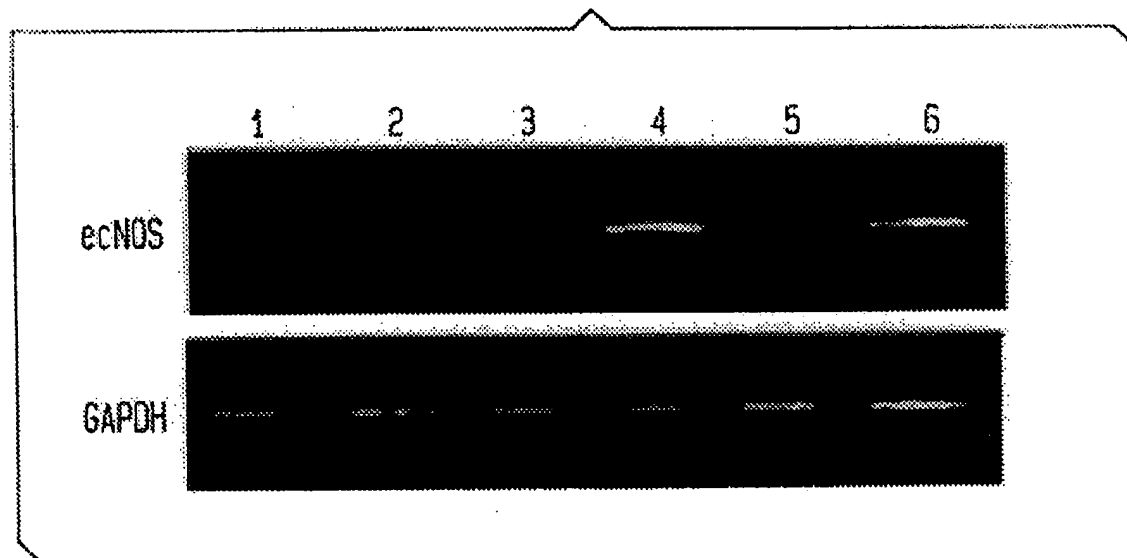
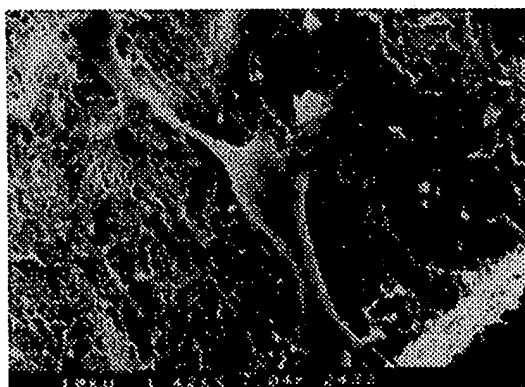
FIG. 4**FIG. 9**

FIG. 6A



FIG. 6B

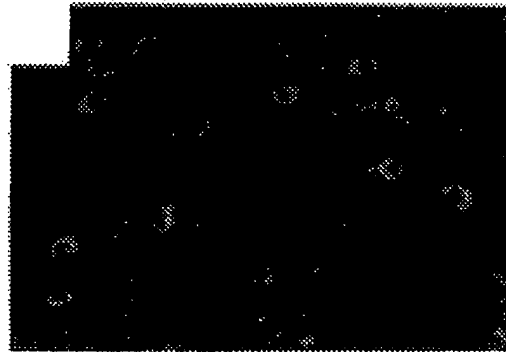
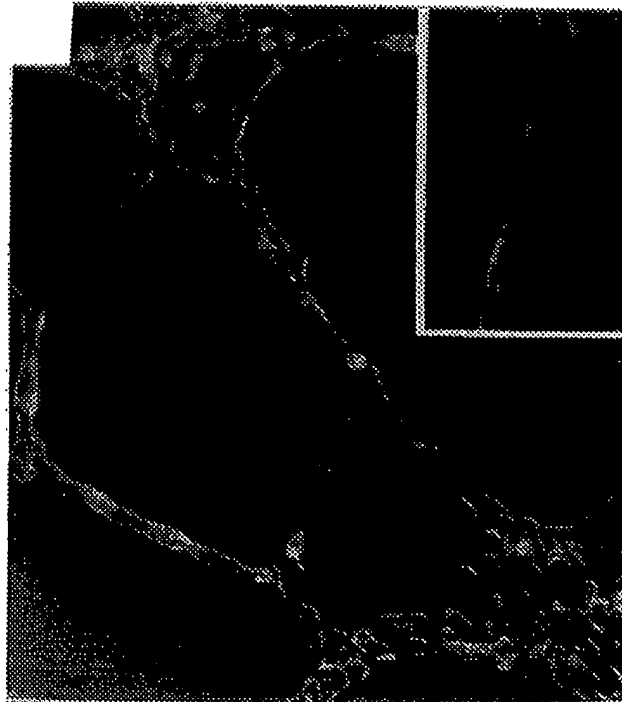


FIG. 6C



FIG. 6D



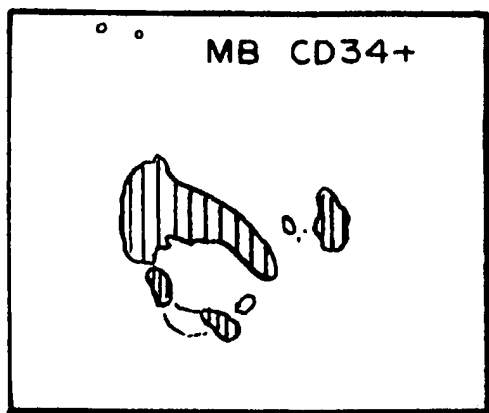


FIG. 8A

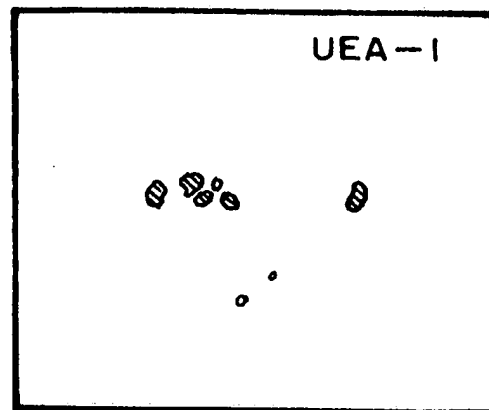


FIG. 8B

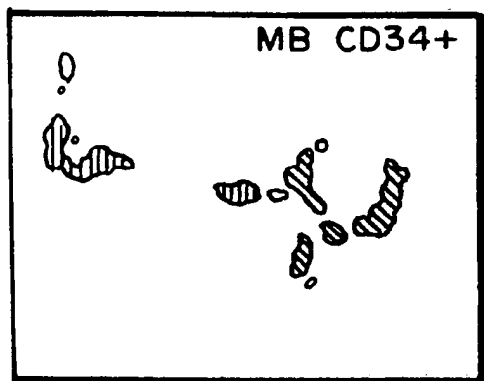


FIG. 8C

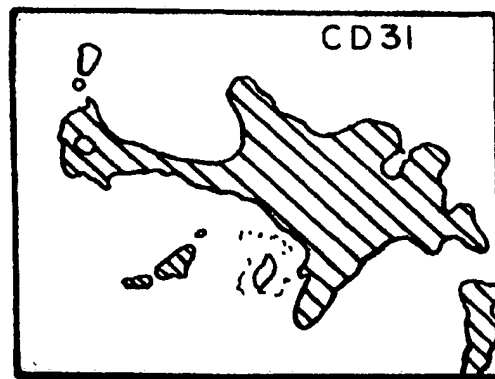


FIG. 8D

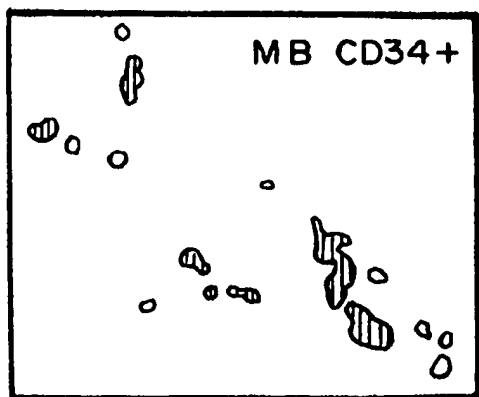


FIG. 8E

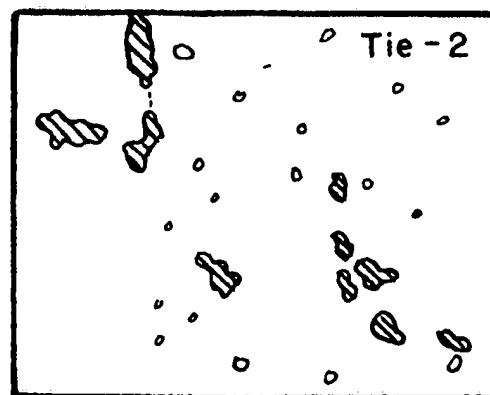


FIG. 8F

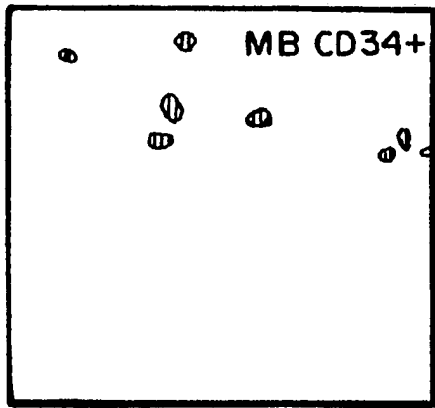


FIG. 8G

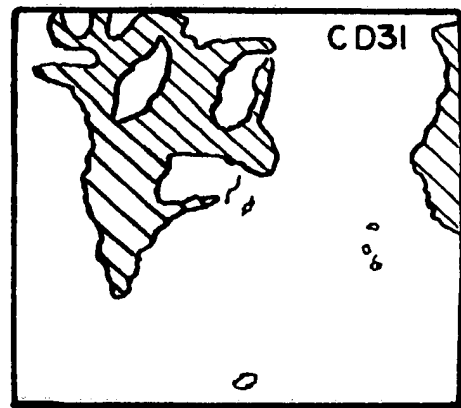


FIG. 8H

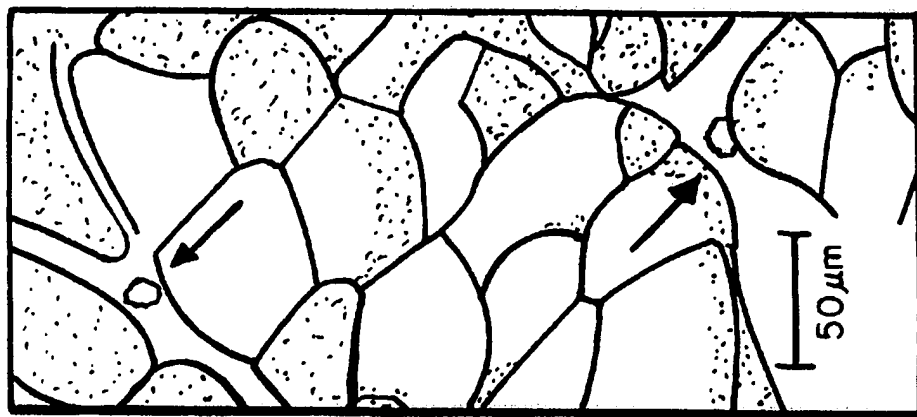


FIG. 8I

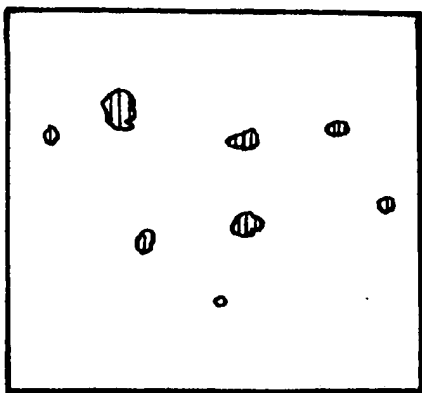


FIG. 8J

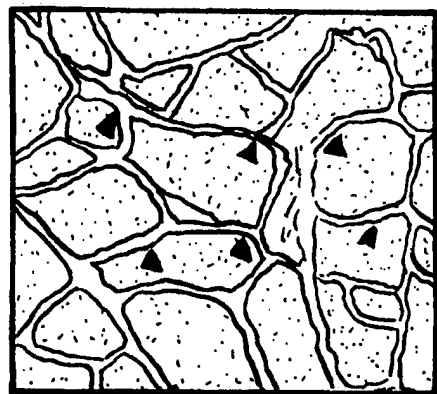


FIG. 8K

METHODS FOR ENHANCING ANGIOGENESIS WITH ENDOTHELIAL PROGENITOR CELLS

BACKGROUND OF THE INVENTION

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products removed from living tissue. Angiogenesis is the process by which new blood vessels are formed, as reviewed, for example, by Folkman and Shing, *J. Biol. Chem.* 267 (16), 10931-10934 (1992). Thus angiogenesis is a critical process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors begin to grow rapidly and metastasize. Because angiogenesis is so critical to these functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

In the adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells can exceed one thousand days. Physiological exceptions in which angiogenesis results in rapid proliferation occurs under tight regulation are found in the female reproduction system and during wound healing.

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. Abnormal angiogenesis occurs when the body loses its control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. On the contrary, excessive blood vessel proliferation may favor tumor growth and spreading, blindness, psoriasis and rheumatoid arthritis.

The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, *N. Engl. J. Med.*, 285:1182-1186 (1971)). Thus, there are instances where a greater degree of angiogenesis is desirable—wound and ulcer healing. Recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et al., *J Clin Invest*, 93:662-70 (1994)).

Conversely, there are also instances, where inhibition of angiogenesis is desirable. For example, many diseases are driven by persistent unregulated angiogenesis. In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. Ocular neovascularization is the most common cause of blindness. Tumor growth and

metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g., drugs (TNP-470), monoclonal antibodies and antisense nucleic acids, are currently being tested. However, new agents that inhibit angiogenesis are need.

Recently, the feasibility of gene therapy for modulating angiogenesis has been demonstrated. For example, promoting angiogenesis in the treatment of ischemia was demonstrated in a rabbit model and in human clinical trials with VEGF using a Hydrogel-coated angioplasty balloon as the gene delivery system. Successful transfer and sustained expression of the VEGF gene in the vessel wall subsequently augmented neovascularization in the ischemic limb (Takeshita, et al., *Laboratory Investigation*, 75:487-502 (1996); Isner, et al., *Lancet*, 348:370 (1996)). In addition, it has been demonstrated that direct intramuscular injection of DNA encoding VEGF into ischemic tissue induces angiogenesis, providing the ischemic tissue with increased blood vessels (U.S. Ser. No. 08/545,998; Tsurumi et al., *Circulation*, In Press).

Alternative methods for regulating angiogenesis are still desirable for a number of reasons. For example, it is believed that native endothelial cell (EC) number and/or viability decreases over time. Thus, in certain patient populations, e.g., the elderly, the resident population of ECs that is competent to respond to administered angiogenic cytokines may be limited.

Moreover, while agents promoting or inhibiting angiogenesis may be useful at one location, they may be undesirable at another location. Thus, means to more precisely regulate angiogenesis at a given location are desirable.

SUMMARY OF THE INVENTION

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiate undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

The present invention provides a method for regulating angiogenesis in a selected patient in need of a change in the rate of angiogenesis at a selected site. The change in angiogenesis necessary may be reduction or enhancement of angiogenesis. This is determined by the disorder to be treated. In accordance with the method of the present invention, an effective amount of an endothelial progenitor cell or modified version thereof to accomplish the desired result is administered to the patient.

In order to reduce undesired angiogenesis, for example, in the treatment of diseases such as rheumatoid arthritis, psoriasis, ocular neovascularization, diabetic retinopathy, neovascular glaucoma, angiogenesis-dependent tumors and tumor metastasis, a modified endothelial cell, having been modified to contain a compound that inhibits angiogenesis, e.g., a cytotoxic compound or angiogenesis inhibitor, can be administered.

To enhance angiogenesis, for example in the treatment of cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia, endothelial progenitor cells are administered. To further enhance angiogenesis an endothelial progenitor cell modified to express an endothelial cell mitogen may be used. Additionally, an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen can further be administered.

In another embodiment, the present invention provides methods of enhancing angiogenesis or treating an injured blood vessel. In accordance with these methods, endothelial progenitor cells are isolated from the patient, preferably from peripheral blood, and readministering to the patient. The patient may also be treated with endothelial cell mitogens to endothelial cell growth. The vessel injury can be the result of balloon angioplasty, deployment of an endovascular stent or a vascular graft.

The present invention also provides a method of screening for the presence of ischemic tissue or vascular injury in a patient. The method involves contacting the patient with a labelled EC progenitor and detecting the labelled cells at the site of the ischemic tissue or vascular injury.

The present invention also includes pharmaceutical products and kit for all the uses contemplated in the methods described herein.

Other aspects of the invention are disclosed infra.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A–1G show cell shape and formation. FIG. 1A shows spindle shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} on fibronectin with standard medium (10). Network formation (1B) and cord-like structures (1C) were observed 48 h after plating co-culture of MB^{CD34+}, labeled with Dil dye (Molecular Probe), and unlabeled MB^{CD34+} (ratio of 1:100) on fibronectin-coated dish. These cords consisted principally of Dil-labeled MB^{CD34+} derived cells (AT^{CD34+}). Beginning 12 h after co-culture, MB^{CD34+} derived cells demonstrated multiple foci of cluster formation (1D, 1E.). AT^{CD34+} sprout from the periphery, while round cells remain in the center and detach from the cluster several days later. After 5 d, uptake of acLDL-Dil (Molecular Probe) was seen in AT^{CD34+} at the periphery but not the center of the cluster (1F, 1G).

FIG. 2 shows the number of AT^{CD34+} 12 h and 3 d after single culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/COL), or fibronectin (CD34+/FN), and MB^{CD34+} on fibronectin (CD34-/FN). AT^{CD34+} yielded significantly higher number of cells at 12 h and 3 d when plated on fibronectin (p<0.05, by ANOVA).

FIG. 3 shows FACS analysis of freshly isolated MB^{CD34+}, AT^{CD34+} after 7 days in culture, and HUVECs. Cells were labeled with FITC using antibodies against CD34, CD31 (Bioscience Resource Project); Flk-1, Tie-2 (Santa Cruz Biotechnology); and CD45. All results were confirmed by triplicate experiments. Shaded area of each box denotes negative antigen gate, white area denotes positive gate. Numbers indicated for individual gates denote percentage of cells determined by comparison with corresponding negative control labeling.

FIG. 4 shows expression of ecNOS mRNA in MB^{CD34+}, MB^{CD34+}, AT^{CD34+}, human coronary smooth muscle cells (HSCMCs) and HUVECs. DNA was reverse transcribed from 1×10^6 cells each. Equal aliquots of the resulting DNA were amplified by PCR (40 cycles) with paired primers (sense/antisense: AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/TGG GGT AGG CAC TTT AGT AGT TCT

CCT AAC, SEQ ID NO:2) to detect ecNOS mRNA. Equal aliquots of the amplified product were analyzed on a 1% agarose gel. Only a single band was observed, corresponding to the expected size (548 bp) for ecNOS. Lane 1=MB^{CD34+}, Lane 2=MB^{CD34+}, Lane 3=AT^{CD34+} after 3 d, Lane 4=AT^{CD34+} after 7 d, Lane 5=HSCMCs, Lane 6=HUVECs.

FIG. 5 is a graph illustrating NO release from AT^{CD34+} was measured with an NO-specific polarographic electrode connected to an NO meter (Iso-NO, World Precision Instruments) (17). Calibration of NO electrode was performed daily before experimental protocol according to the following equation: $2\text{KNO}_2 + 2\text{KI} + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{NO} + \text{I}_2 + 2\text{H}_2\text{O} + 2\text{K}_2\text{SO}_4$. standard calibration curve was obtained by adding graded concentrations of KNO₂ (0–500 nmol/L) to calibration solution containing KI and H₂SO₄. Specificity of the electrode to NO was previously documented by measurement of NO from authentic NO gas (18). AT^{CD34+} cultured in 6-well plate were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments) to maintain temperature between 35 and 37° C. For NO measurement, sensor probe was inserted vertically into the wells, and the tip of the electrode remained 2 mm under the surface of the solution. Measurement of NO, expressed as pmol/10⁵ cells, was performed in a well with incremental doses of VEGF (1, 10, 100 ng/ml) and Ach (0.1, 1, 10 μ M). HUVECs and bovine aortic ECs were employed as positive controls. For negative control, HSCMCs, NO was not detectable. All values reported represent means of 10 measurements for each group.

FIGS. 6A–6D show co-culture of MB^{CD34+} with HUVECs. Freshly isolated MB^{CD34+} were labeled with Dil dye and plated on a confluent HUVEC monolayer attached to a fibronectin-coated chamber slide at a density of 278 cells/mm² (Nunc). Differentiation of MB^{CD34+} into spindle shaped attaching cells (AT^{CD34+}) (red fluorescence) was observed among HUVECs within 12 h (6A). The AT^{CD34+} number increased on monolayer for 3 d (6B), while mesh-work structures were observed in some areas (6C). Three days after co-culture, both cells were re-seeded on Matrigel (Becton Dickinson)-coated slides and within 12 h disclosed capillary network formation, consisting of Dil-labeled AT^{CD34+} and HUVECs (6D).

FIG. 7 shows the effect of activated ECs and VEGF on MB^{CD34+} differentiation was investigated by pretreatment of HUVEC with TNF- α (20 ng/ml) for 12 h, and/or incubation of AT^{CD34+}/HUVEC co-culture with VEGF (50 ng/ml).

FIGS. 8A–8K show sections retrieved from ischemic hindlimb following in vivo administration of heterologous (FIGS. 8A–8H), homologous (8I), and autologous (8J, 8K) EC progenitors. (8A, 8B) Red fluorescence in small inter-muscular artery 6 wks after injection of Dil-labeled MB^{CD34+}. Green fluorescence denotes EC-specific lectin UEA-1. (8C) Dil (red) and CD31 (green) in capillaries between muscles, photographed through double filter 4 wks after Dil-labeled MB^{CD34+} injection. (8D) Same capillary structure as in (C), showing CD31 expression by MB^{CD34+} which have been incorporated into host capillary structures expressing CD31. (8E, 8F) Immunostaining 2 wks after MB^{CD34+} injection shows capillaries comprised of Dil-labeled MB^{CD34+} derived cells expressing tie-2 receptor (green fluorescence). Most MB^{CD34+} derived cells are tie-2 positive, and are integrated with some tie-2 positive native (host) capillary cells identified by absence of red fluorescence. (8G, 8H) Two wks after injection of Dil-labeled MB^{CD34+}. Although isolated MB^{CD34+} derived cells (red) can be observed between muscles, but these cells do not express CD31.

(8I) Immunohistochemical, β -galactosidase staining of muscle harvested from ischemic limb of B6, 129 mice 4 wks following administration of MB^{Flk-1+} isolated from β -galactosidase transgenic mice. Cells overexpressing β -galactosidase (arrows) have been incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 antibody and BS-1 lectin.

(8J,8K) Sections of muscles harvested from rabbit ischemic hindlimb 4 wks after administration of autologous MB^{CD34+}. Dil fluorescence (J) indicates localization of MB^{CD34+} derived cells in capillaries seen in phase contrast photomicrograph (8K). Each scale bar indicates 50 μ m.

FIG. 9 is a photograph from a scanning electron microscope showing that EC progenitors had adhered to the denuded arterial surface and assumed a morphology suggestive of endothelial cells.

DETAILED DESCRIPTION OF THE INVENTION

We have now discovered a means to regulate angiogenesis, to promote angiogenesis in certain subject populations, and to more precisely target certain tissues. These methods all involve the use of endothelial cell progenitors. One preferred progenitor cell is an angioblast.

Post-natal neovascularization is believed to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells (ECs) derived from pre-existing native blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to formation of embryonic blood vessels from EC progenitors (2).

In contrast to angiogenesis, vasculogenesis typically begins as a cluster formation, or blood island, comprised of EC progenitors (e.g. angioblasts) at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this intimate and predictable spatial association, such EC progenitors and HSCs share certain common antigenic determinants, including flk-1, tie-2, and CD-34. Consequently, these progenitor cells have been interpreted to derive from a common hypothetical precursor, the hemangioblast (3,4).

The demonstration that transplants of HSCs derived from peripheral blood can provide sustained hematopoietic recovery constitutes inferential evidence for circulating stem cells. (5). This observation is now being exploited clinically as an alternative to bone marrow transplantation.

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiated undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

In accordance with the present invention, EC progenitors can be used in a method for regulating angiogenesis, i.e., enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targeting specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis

modulator, e.g. anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce reendothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

In one preferred embodiment the EC cells can be used alone to potentiate a patient for angiogenesis. Some patient population, typically elderly patients, may have either a limited number of ECs or a limited number of functional ECs. Thus, if one desires to promote angiogenesis, for example, to stimulate vascularization by using a potent angiogenesis promotor such as VEGF, such vascularization can be limited by the lack of ECs. However, by administering the EC progenitors one can potentiate the vascularization in those patients.

Accordingly, the present method permits a wide range of strategies designed to modulate angiogenesis such as promoting neovascularization of ischemic tissues (24). EC mitogens such as VEGF and bFGF, for example, have been employed to stimulate native ECs to proliferate, migrate, remodel and thereby form new sprouts from parent vessels (25). A potentially limiting factor in such therapeutic paradigms is the resident population of ECs that is competent to respond to administered angiogenic cytokines. The finding that NO production declines as a function of age (26) may indicate a reduction in EC number and/or viability that could be addressed by autologous EC grafting. The success demonstrated to date with autologous grafts of HSCs derived from peripheral blood (5) supports the clinical feasibility of a "supply side" approach to therapeutic angiogenesis. The in vivo data set forth herein indicate that autologous EC transplants are feasible, and the in vitro experiments indicate that EC progenitors (MB^{CD34+} derived ECs) can be easily manipulated and expanded ex vivo.

Our discovery that these EC progenitors home to foci of angiogenesis makes these cells useful as autologous vectors for gene therapy and diagnosis of ischemia or vascular injury. For example, these cells can be utilized to inhibit as well as augment angiogenesis. For anti-neoplastic therapies, for example, EC progenitors can be transfected with or coupled to cytotoxic agents, cytokines or co-stimulatory molecules to stimulate an immune reaction, other anti-tumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by prior transfection of EC progenitors to achieve constitutive expression of angiogenic cytokines and/or selected matrix proteins (27). In addition, the EC progenitors may be labelled, e.g., radiolabelled, administered to a patient and used in the detection of ischemic tissue or vascular injury.

EC progenitors may be obtained from human mononuclear cells obtained from peripheral blood or bone marrow of the patient before treatment. EC progenitors may also be obtained from heterologous or autologous umbilical cord blood. Peripheral blood is preferred due to convenience. The leukocyte fraction of peripheral blood is most preferred. EC progenitors may be isolated using antibodies that recognize EC progenitor specific antigens on immature human hematopoietic progenitor cells (HSCs). For example, CD34 is commonly shared by EC progenitor and HSCs. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (6). It is also expressed by many, including most activated, ECs in the adult (7). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (8), is also expressed by both early HSCs and ECs, but ceases to be expressed in the course of hematopoietic differentiation (9).

To obtain the EC progenitors from peripheral blood about 5 ml to about 500 ml of blood is taken from the patient. Preferably, about 50 ml to about 200 ml of blood is taken.

EC progenitors can be expanded *in vivo* by administration of recruitment growth factors, e.g., GM-CSF and IL-3, to the patient prior to removing the progenitor cells.

Methods for obtaining and using hematopoietic progenitor cells in autologous transplantation are disclosed in U.S. Pat. No. 5,199,942, the disclosure of which is incorporated by reference.

Once the progenitor cells are obtained by a particular separation technique, they may be administered to a selected patient to treat a number of conditions including, for example, unregulated angiogenesis or blood vessel injury. The cells may also be stored in cryogenic conditions. Optionally, the cells may be expanded *ex vivo* using, for example, the method disclosed by U.S. Pat. No. 5,541,103, the disclosure of which is incorporated by reference.

The progenitor cells are administered to the patient by any suitable means, including, for example, intravenous infusion, bolus injection, and site directed delivery via a catheter. Preferably, the progenitor cells obtained from the patient are readministered. Generally, from about 10^6 to about 10^{18} progenitor cells are administered to the patient for transplantation.

Depending on the use of the progenitor cells, various genetic material may be delivered to the cell. The genetic material that is delivered to the EC progenitors may be genes, for example, those that encode a variety of proteins including anticancer agents. Such genes include those encoding various hormones, growth factors, enzymes, cytokines, receptors, MHC molecules and the like. The term "genes" includes nucleic acid sequences both exogenous and endogenous to cells into which a virus vector, for example, a pox virus such as swine pox containing the human TNF gene may be introduced. Additionally, it is of interest to use genes encoding polypeptides for secretion from the EC progenitors so as to provide for a systemic effect by the protein encoded by the gene. Specific genes of interest include those encoding TNF, TGF- α , TGF- β , hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12 etc., GM-CSF, G-CSF, M-CSF, human growth factor, co-stimulatory factor B7, insulin, factor VIII, factor IX, PDGF, EGF, NGF, IL-1ra, EPO, β -globin, EC mitogens and the like, as well as biologically active muteins of these proteins. The gene may further encode a product that regulates expression of another gene product or blocks one or more steps in a biological pathway. In addition, the gene may encode a toxin fused to a polypeptide, e.g., a receptor ligand, or an antibody that directs the toxin to a target, such as a tumor cell. Similarly, the gene may encode a therapeutic protein fused to a targeting polypeptide, to deliver a therapeutic effect to a diseased tissue or organ.

The cells can also be used to deliver genes to enhance the ability of the immune system to fight a particular disease or tumor. For example, the cells can be used to deliver one or more cytokines (e.g., IL-2) to boost the immune system and/or one or more antigens.

These cells may also be used to selectively administer drugs, such as an antiangiogenesis compound such as O-chloroacetyl carbamoyl fumagillol (TNP-470). Preferably the drug would be incorporated into the cell in a vehicle such as a liposome, a timed released capsule, etc. The EC progenitor would then selectively home in on a site of active angiogenesis such as a rapidly growing tumor where the compound would be released. By this method, one can reduce undesired side effects at other locations.

In one embodiment, the present invention may be used to enhance blood vessel formation in ischemic tissue, i.e., a tissue having a deficiency in blood as the result of an ischemic disease. Such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

If it is desirable to further enhance angiogenesis, endothelial cell mitogens may also be administered to the patient in conjunction with, or subsequent to, the administration of the EC progenitor cells. Endothelial cell mitogens can be administered directly, e.g., intra-arterially, intramuscularly, or intravenously, or nucleic acid encoding the mitogen may be used. See, Baffour, et al., supra (bFGF); Pu, et al., *Circulation*, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., supra (bFGF); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851-855 (1989) (VEGF); (Takeshita, et al., *Circulation*, 90:228-234 (1994)).

The nucleic acid encoding the EC mitogen can be administered to a blood vessel perfusing the ischemic tissue or to a site of vascular injury via a catheter, for example, a hydrogel catheter, as described by U.S. Ser. No. 08/675,523, the disclosure of which is herein incorporated by reference. The nucleic acid also can be delivered by injection directly into the ischemic tissue using the method described in U.S. Ser. No. 08/545,998.

As used herein the term "endothelial cell mitogen" means any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing endothelial cell growth. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF- α and TGF- β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF- α), hepatocyte growth factor (HGF), insulin like growth factor (IGF), erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992) and Symes, et al., *Current Opinion in Lipidology*, 5:305-312 (1994). Muteins or fragments of a mitogen may be used as long as they induce or promote EC cell growth.

Preferably, the endothelial cell mitogen contains a secretory signal sequence that facilitates secretion of the protein. Proteins having native signal sequences, e.g., VEGF, are preferred. Proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The nucleotide sequence of numerous endothelial cell mitogens, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g., PCR amplification. A DNA encoding VEGF is disclosed in U.S. Pat. No. 5,332,671, the disclosure of which is herein incorporated by reference.

In certain situations, it may be desirable to use nucleic acids encoding two or more different proteins in order to optimize the therapeutic outcome. For example, DNA encoding two proteins, e.g., VEGF and bFGF, can be used,

and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

The term "effective amount" means a sufficient amount of compound, e.g. nucleic acid delivered to produce an adequate level of the endothelial cell mitogen, i.e., levels capable of inducing endothelial cell growth and/or inducing angiogenesis. Thus, the important aspect is the level of mitogen expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be under the control of a factor that results in extremely high levels of expression, e.g., tat and the corresponding tar element.

The EC progenitors may also be modified ex vivo such that the cells inhibit angiogenesis. This can be accomplished, for example, by introducing DNA encoding angiogenesis inhibiting agents to the cells, using for example the gene transfer techniques mentioned herein. Angiogenesis inhibiting agents include, for example, proteins such as thrombospondin (Dameron et al., *Science* 265:1582-1584 (1994)), angiostatin (O'Reilly et al., *Cell* 79:315-328 (1994), IFN-alpha (Folkman, J. *Nature Med.* 1:27-31 (1995)), transforming growth factor beta, tumor necrosis factor alpha, human platelet factor 4 (PF4); substances which suppress cell migration, such as proteinase inhibitors which inhibit proteases which may be necessary for penetration of the basement membrane, in particular, tissue inhibitors of metalloproteinase TIMP-1 and TIMP-2; and other proteins such as protamine which has demonstrated angiostatic properties, decoy receptors, drugs such as analogues of the angioinhibitor fumagillin, e.g., TNP-470 (Ingber et al., *Nature* 348:555-557 (1990)), antibodies or antisense nucleic acid against angiogenic cytokines such as VEGF. Alternatively, the cells may be coupled to such angiogenesis inhibiting agent.

If the angiogenesis is associated with neoplastic growth the EC progenitor cell may also be transfected with nucleic acid encoding, or coupled to, anti-tumor agents or agents that enhance the immune system. Such agents include, for example, TNF, cytokines such as interleukin (IL) (e.g., IL-2, IL-4, IL-10, IL-12), interferons (IFN) (e.g., IFN-γ) and co-stimulatory factor (e.g., B7). Preferably, one would use a multivalent vector to deliver, for example, both TNF and IL-2 simultaneously.

The nucleic acids are introduced into the EC progenitor by any method which will result in the uptake and expression of the nucleic acid by the cells. These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, gene gun, etc.

Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-based viruses. One

preferred HIV-based viral vector comprises at least two vectors wherein the gag and pol genes are from an HIV genome and the env gene is from another virus. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as a herpes simplex I virus (HSV) vector [Geller, A. I. et al., *J. Neurochem.* 64: 487 (1995); Lim, F., et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A. I. et al., *Proc Natl. Acad. Sci. U.S.A.* 90:7603 (1993); Geller, A. I., et al., *Proc Natl. Acad. Sci. USA* 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet* 3: 219 (1993); Yang, et al., *J. Virol.* 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplit, M. G., et al., *Nat. Genet.* 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, CaPO₄ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, viral vectors and use of the "gene gun".

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can be expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a tat gene and tar element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β-lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

If desired, the preselected compound, e.g. a nucleic acid such as DNA may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R. A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584

(1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

The effective dose of the nucleic acid will be a function of the particular expressed protein, the target tissue, the patient and his or her clinical condition. Effective amount of DNA are between about 1 and 4000 μ g, more preferably about 1000 and 2000, most preferably between about 2000 and 4000.

Alternatively, the EC progenitors may be used to inhibit angiogenesis and/or neoplastic growth by delivering to the site of angiogenesis a cytotoxic moiety coupled to the cell. The cytotoxic moiety may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal or plant origin, or an enzymatically active polypeptide chain or fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof are preferred and are exemplified by diphtheria toxin A fragment, non-binding active fragments of diphtheria toxin, exotoxin A (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alphasarcin, certain Aleurites fordii proteins, certain Dianthin proteins, Phytolacca americana proteins (PAP, PAPII and PAP-S), Momordica charantia inhibitor, curcin, crotin, Saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. Ricin A chain, *Pseudomonas aeruginosa* exotoxin A and PAP are preferred.

Conjugates of the EC progenitors and such cytotoxic moieties may be made using a variety of coupling agents. Examples of such reagents are N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters such as dimethyl adeipimide HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

The enzymatically active polypeptide of the toxins may be recombinantly produced. Recombinantly produced ricin toxin A chain (rRTA) may be produced in accordance with the methods disclosed in PCT W085/03508 published Aug. 15, 1985. Recombinantly produced diphtheria toxin A chain and non-binding active fragments thereof are also described in PCT W085/03508 published Aug. 15, 1985.

The methods of the present invention may be used to treat blood vessel injuries that result in denuding of the endothelial lining of the vessel wall. For example, primary angioplasty is becoming widely used for the treatment of acute myocardial infarction. In addition, endovascular stents are becoming widely used as an adjunct to balloon angioplasty. Stents are useful for rescuing a sub-optimal primary result as well as for diminishing restenosis. To date, however, the liability of the endovascular prosthesis has been its susceptibility to thrombotic occlusion in approximately 3% of patients with arteries 3.3 mm or larger. If patients undergo stent deployment in arteries smaller than this the incidence of sub-acute thrombosis is even higher. Sub-acute thrombosis is currently prevented only by the aggressive use of anticoagulation. The combination of vascular intervention and intense anticoagulation creates significant risks with regard to peripheral vascular trauma at the time of the stent/angioplasty procedure. Acceleration of reendothelialization by administration of EC progenitors to a patient undergoing, or subsequent to, angioplasty and/or stent deployment can stabilize an unstable plaque and prevent re-occlusion.

The method of the present invention may be used in conjunction with the method for the treatment of vascular injury disclosed in PCT/US96/15813.

In addition, the methods of the present invention may be used to accelerate the healing of graft tissue, e.g., vascular grafts.

The present invention also includes pharmaceutical products for all the uses contemplated in the methods described herein. For example, there is a pharmaceutical product, comprising nucleic acid encoding an endothelial cell mitogen and EC progenitors, in a physiologically acceptable administrable form.

The present invention further includes a kit for the in vivo systemic introduction of an EC progenitor and an endothelial cell mitogen or nucleic acid encoding the same into a patient. Such a kit includes a carrier solution, nucleic acid or mitogen, and a means of delivery, e.g., a catheter or syringe. The kit may also include instructions for the administration of the preparation.

All documents mentioned herein are incorporated by reference herein in their entirety.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Method and Materials

Human peripheral blood was obtained using a 20 gauge intravenous catheter, discarding the first 3 ml. Leukocyte fraction of blood was obtained by Ficoll density gradient centrifugation and plated on plastic tissue culture for 1 hr to avoid contamination by differentiated adhesive cells.

Fluorescent activated cell sorting (FACS) was carried out with $>1 \times 10^6$ CD34 positive and negative mononuclear blood cells (MB^{CD34+}, MB^{CD34-}). Cells were analyzed with Becton-Dickinson FACS sorter and the lysis II analysis program using antibodies to CD34 (Bioscience).

M-199 medium with 20% FBS and bovine brain extract (Clonetics) was used as standard medium for all cell culture experiments.

C57BL/6Jx129/SV background male mice (Hirlean), 3 mo old and 20-30 g, were used in these experiments (n=24). Animals were anesthetized with 160 mg/kg intraperitoneally of pentobarbital. The proximal end of one femoral artery and distal portion of the corresponding saphenous artery were ligated, following which the artery, as well as all side-branches, were dissected free and excised. (All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee.)

New Zealand White rabbits (3.8-4.2 kg, n=4, Pine Acre Rabbitry) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2 mg/kg). After a longitudinal incision, the femoral artery was dissected free along its entire length; all branches of the femoral artery were also dissected free. After ligating the popliteal and saphenous arteries distally, the external iliac artery proximally and all femoral arterial branches, the femoral artery was completely excised (23). Isolation and Analysis

CD34 positive mononuclear blood cells (MB^{CD34+}) were isolated from peripheral blood by CD34 antibody-coated magnetic beads (Dynal) as described above.

FACS analysis indicated that $15.9 \pm 3.3\%$ of selected cells versus $<0.1\%$ of the remaining cells expressed CD34. Depleted (MB^{CD34-}) cells were used as controls. Flk-1 antibody was used for magnetic bead selection of Flk-1 positive mononuclear blood cells (MB^{Flk1+}).

MB^{CD34+} and MB^{CD34-} were plated separately in standard medium on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of $1 \times 10^3/\text{mm}^2$, a limited number of MB^{CD34+} attached, and became spindle shaped and proliferated for 4 wks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (FIG. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (FIG. 2). Attached cells were observed only sporadically among cultures of MB^{CD34-}, including cells followed for up to 4 wks on fibronectin-coated plates.

To confirm that spindle-shaped cells were derived from CD34 positive cells, MB^{CD34+} were labeled with the fluorescent dye, Dil, and co-plated with unlabeled MB^{CD34-} on fibronectin at an overall density of $5 \times 10^3/\text{mm}^2$; ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^{CD34+}, 99% MB^{CD34-}). Seven days later, Dil-labeled cells derived from MB^{CD34+}, initially accounting for only 1% of blood cells, accounted for $60.3 \pm 4.7\%$ of total attaching cells analyzed by FACS. Co-incubation with MB^{CD34-} increased proliferation to $>10 \times$ MB^{CD34+} plated alone at a cell density of $5 \times 10^3/\text{mm}^2$ cell ($d = 131.3 \pm 26.8$ vs $9.7 \pm 3.5/\text{mm}^2$). MB^{CD34+}/MB^{CD34-} co-cultures also enhanced MB^{CD34+} differentiation, including formation of cellular networks and tube-like structures on fibronectin-coated plates (FIGS. 1B,C). These structures consisted principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1C). Moreover, within 12 h of co-culture, multiple cluster formations were observed (FIG. 1D), consisting principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1E). These clusters were comprised of round cells centrally, and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which induced ECs and gave rise to vascular structures in vitro (3). AT^{CD34+} at the cluster periphery were shown to take up Dil-labeled acetylated LDL, characteristic of EC lineage (13), whereas the round cells comprising the center of cluster did not (FIGS. 1F,G); the latter detached from the cluster several days later. Similar findings were observed in the experiments using MB^{Flk1+}.

Expression of Leukocyte and EC Markers

To further evaluate progression of MB^{CD34+} to an EC-like phenotype, cells were assayed for expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} versus AT^{CD34+} cultured at densities of 1×10^3 cell/ mm^2 for 7 days were incubated with fluorescent-labeled antibodies and analyzed by FACS (FIG. 3). Leukocyte common antigen, CD45, was identified on 94.1% of freshly isolated cells, but was essentially lost by 7 d in culture (FIG. 3). Augmented expression of UEA-1, CD34, CD31, Flk-1, Tie-2 and E-selectin—all denoting EC lineage (14)—was detected among AT^{CD34+} after 7 days in culture, compared to freshly isolated MB^{CD34+}. CD68 expression, suggesting monocyte/macrophage lineage, was limited to $6.0 \pm 2.4\%$ cells.

Expression of Factor VIII, UEA-1, CD31, eNOS, and E-selectin was also documented by immunohistochemistry for AT^{CD34+} after 7 days culture (data not shown). After 3, 7, and 14 days in culture, more than 80% AT^{CD34+} took up Dil-labeled acLDL (13).

ECs uniquely express endothelial constitutive nitric oxide synthase (eNOS). Accordingly, MB^{CD34+}, MB^{CD34-} and AT^{CD34+} were investigated for expression of eNOS by RT-PCR (15). eNOS mRNA was not detectable among MB^{CD34-} and was present at very low levels in freshly isolated MB^{CD34+} (FIG. 4). In AT^{CD34+} cultured for 7 d, however, eNOS mRNA was markedly increased (FIG. 5).

Functional evidence of eNOS protein in AT^{CD34+} was documented by measurement of nitric oxide in response to the EC-dependent agonist, acetylcholine (ACh), and the EC-specific mitogen, vascular endothelial growth factor (VEGF) (16) (FIG. 5); the latter parenthetically constitutes evidence for a functional Flk-1 receptor as well among AT^{CD34+}.

Cell-Cell Interaction

Cell-cell interaction is considered to play a decisive role in cell signaling, differentiation, and proliferation during hematopoiesis (19) and angiogenesis (20). To study the impact of MB^{CD34+} interaction with mature ECs on the differentiation of MB^{CD34+} into an EC-like phenotype, Dil-labeled MB^{CD34+} were plated on a confluent HUVEC monolayer. Adherent, labeled cells were found throughout the culture within 12 h (FIG. 6A), and increased in number for up to 3 d (FIG. 6B). When incubated with 50 ng/ml VEGF and 10 ng/ml bFGF, a meshwork of cord-like structures comprised of both Dil-labeled and unlabeled cells could be seen within 3 d after co-culture (FIG. 6C). Both cell types were then re-seeded on Matrigel (Becton Dickinson) coated slides and within 12 h demonstrated formation of capillary networks comprised of Dil-labeled MB^{CD34+} derived cells and HUVECs (FIG. 6D). To facilitate cell-cell interaction, HUVECs were pre-treated with TNF- α (21), resulting in increased numbers of AT^{CD34+} (FIG. 6E); synergistic augmentation was observed upon co-incubation with VEGF. Identically treated co-cultures of HUVECs and Dil-labeled MB^{CD34+} yielded desquamated labeled cells and/or no cords. Similar findings were observed when EC precursors were isolated using MB^{Flk1+}.

In Vivo Angiogenesis

Previous studies have established that ECs constitute the principal cell responsible for in vivo angiogenesis (1). To determine if MB^{CD34+} can contribute to angiogenesis in vivo, we employed two previously characterized animal models of hindlimb ischemia. For administration of human MB^{CD34+}, C57BL/6Jx129/SV background athymic nude mice were employed to avoid potential graft-versus host complications. Two days later, when the limb was severely ischemic, mice were injected with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34-} via the tail vein. Histologic sections of limbs examined 1, 2, 4, and 6 wks later for the presence of Dil labeled cells revealed numerous Dil-labeled cells in the neo-vascularized ischemic hindlimb. Labeled cells were more numerous in MB^{CD34+} versus MB^{CD34-} injected mice, and almost all labeled cells appeared to be integrated into capillary vessel walls (FIG. 8A,C,E,G).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34-} injected mice. Dil labeled cells were also consistently co-labeled with immunostains for UEA-1 lectin (FIG. 8B), CD31 (FIG. 8D), and Tie-2 (FIG. 8F). In contrast, in hindlimb sections from mice injected with MB^{CD34-}, labeled cells were typically found in stroma near capillaries, but did not form part of the vessel wall, and did not label with UEA-1 or anti-CD31 antibodies (FIG. 8G,H).

A transgenic mouse overexpressing β -galactosidase was then used to test the hypothesis that homologous grafts of EC progenitors could contribute to neovascularization in vivo. Flk-1 cell isolation was used for selection of EC progenitors due to lack of a suitable anti-mouse CD34 antibody. Approximately 1×10^4 MB^{Flk1+} were isolated from whole blood of 10 β -galactosidase transgenic mice with B6, 129 genetic background. MB^{Flk1+} or the same number of MB^{Flk1-} were injected into B6, 129 mice with hindlimb ischemia of 2 days duration. Immunostaining of ischemic

tissue for β -galactosidase, harvested 4 wks after injection, demonstrated incorporation of cells expressing β -galactosidase in capillaries and small arteries (FIG. 8I); these cells were identified as ECs by staining with anti-CD31 antibody and BS-1 lectin.

Finally, in vivo incorporation of autologous MB^{CD34+} into foci of neovascularization was tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand white rabbits immediately prior to surgical induction of unilateral hindlimb ischemia. Immediately following completion of the operative procedure, freshly isolated autologous Dil-labeled MB^{CD34+} were re-injected into the ear vein of the same rabbit from which the blood had been initially obtained. Four wks after ischemia, histologic sections of the ischemic limbs were examined. Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb, incorporated into capillaries and consistently expressing CD31 and UEA-1 (FIG. 8J,K).

Consistent with the notion that HSCs and ECs are derived from a common precursor, our findings suggest that under appropriate conditions, a subpopulation of MB^{CD34+} or MB^{Fik1+} can differentiate into ECs in vitro. Moreover, the in vivo results suggest that circulating MB^{CD34+} or MB^{Fik1+} in the peripheral blood may constitute a contingency source of ECs for angiogenesis. Incorporation of in situ differentiating EC progenitors into the neovasculature of these adult species is consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2,3). The fact that these cells do not incorporate into mature blood vessels not undergoing angiogenesis suggests that injury, ischemia, and/or active angiogenesis are required to induce in situ differentiation of MB^{CD34+} to ECs.

EXAMPLE II

EC Progenitors Augment Reendothelialization

Following balloon injury, a denuded rat carotid artery was immediately excised and placed in HUVEC medium, and Dil labeled CD34+ EC progenitor cells were seeded onto the artery. After 1 wk, the artery was washed with PBS to remove non-adherent cells. Consistent with the ability of CD34+ cells to differentiate into filtrating cells, Dil labeled cells were found within the smooth muscle cell layer of the artery.

Scanning electron microscopy of the intimal surface, however, showed that Dil-labeled cells also had adhered to the denuded arterial surface, assuming a morphology suggestive of ECs (FIG. 9). Dil labeled cells also incorporated into the capillary-like sprouts at the bare ends of the excised arterial segment, suggesting that CD34+ cells may be capable of participating in angiogenesis as well.

To determine if exogenously administered CD34+ EC progenitor cells can contribute to reendothelialization of a denuded arterial surface in vivo, freshly isolated human CD34+ or CD34- cells were Dil labeled and seeded onto a denuded carotid artery of a nude rat. Following balloon denudation, 1.0x10⁶ labeled cells in PBS was introduced into the denuded artery via a 22 G catheter, which remained in the artery for 30 min before the needle was withdrawn. The external carotid artery was then ligated, the common and internal carotid arterial ligatures removed, and the incision closed. The next day the rat was anesthetized and the vasculature perfusion fixed with Histo Choice (Amresco). The denuded arterial segment was excised and examined for the presence of adherent Dil labeled cells, which were identified in arteries seeded with CD34+ cells, but not CD34- cells.

The following references are cited throughout the specification. All documents mentioned herein are incorporated herein by reference.

- (1) J. Folkman, Y. Shing, *J. Biol. Chem.* 267, 10931 (1992); W. Schaper, M. D. Brahander, P. Lewi, *Circ. Res.* 28, 671 (1971); W. Risau, *FASEB J.* 9, 926 (1995).
- (2) W. Risau, H. Sariola, H-G. Zerwes, J. Sasse, P. Ekblom, et al, *Development* 102, 471 (1988); L. Pardanaud, C. Altman, P. Kitos, F. Dieterien-Lievre, *Development* 105, 473 (1989).
- (3) I. Flamme, W. Risau, *Development* 116, 435 (1992).
- (4) W. His, *Abhandl. K. S. Ges. Wiss. Math. Phys.* 22, 171 (1900); M. Weiss, S. H. Orkin, *J. Clin. Invest.* 97, 591 (1996).
- (5) W. Brugger, S. Heimfeld, R. J. Berenson, R. Mertelsmann, L. Kanz, *N. Engl. J. Med.* 333, 283 (1995); A. Kessinger, J. O. Armitage, *Blood* 77, 211 (1991); W. P. Sheridan, C. G. Begley, C. Juttener, *Lancet* 339, 640 (1992); E. J. Shpall, R. B. Jones, S. I. Bearman, *J. Clin. Oncol.* 12, 28 (1994).
- (6) C. I. Civin, L. C. Strauss, C. Brovall, M. J. Frackler, J. F. Schwartz, et al, *J. Immunol* 133, 157 (1984); F. Katz, R. W. Tindle, D. R. Sutherland, M. D. Greaves, *Leuk. Res.* 9, 191 (1985); R. G. Andrews, J. W. Singer, I. D. Bernstein, *Blood* 67, 842 (1986).
- (7) J. Fina, H. V. Molgard, D. Robertson, N. J. Bradley, P. D. Managhan, et al, *Blood* 75, 2417 (1990); D. Soligo, D. Delia, A. Oriani, G. Cattoretti, A. Orazi, et al, *Leukemia* 5, 1026 (1991); A. Ito, S. Nomura, S. Hirota, T. Suda, Y. Kitamura, *Lab. Invest.* 72, 532 (1995).
- (8) C. deVries, J. A. Escobedo, H. Ueno, K. Houck, N. Ferrara, et al, *Science* 255, 989 (1992); B. I. Terman, M. Dougher-Vermazen, M. E. Carrion, D. Dimitrov, D. C. Armellino, et al, *Biochem. Biophys. Res. Commun.* 187, 1579 (1992); F. Shalaby, J. Rossant, T. P. Yamaguchi, M. Gertsenstein, X-F. Wu, et al, *Nature* 376, 62 (1995).
- (9) W. Matthews, C. T. Jordan, M. Gavin, N. A. Jenkins, N. G. Copeland, et al, *Proc. Natl. Acad. Sci. USA.* 88, 9026 (1991); B. Millauer, S. Witzigmann-Voos, H. Schnurch, R. Martinez, N. P. H. Moller, et al, *Cell* 72, 835 (1993); T. P. Yamaguchi, D. J. Dumont, R. A. Conlon, M. L. Breitman, J. Rossant, *Development* 118, 489 (1993).
- (13) J. C. Voyta, D. P. Via, C. E. Butterfield, B. R. Zetter, *J. Cell Biol.* 99, 2034 (1984).
- (14) B. Millauer, S. Witzigmann-Voos, H. Schnurch, R. Martinez, N. P. H. Moller, et al, *Cell* 72, 835 (1993); T. P. Yamaguchi, D. J. Dumont, R. A. Conlon, M. L. Breitman, J. Rossant, *Development* 118, 489 (1993); M. Miettinen, H. Holthofer, V. P. Lehto, A. Miettinen, I. Virtanen, *Am. J. Clin. Pathol.* 79, 32 (1983); E. A. Jaffe, R. L. Nachman, C. G. Becker, C. R. Minick, *J. Clin. Invest.* 52, 2745 (1973); P. J. Newman, M. C. Berndt, J. Gorski, G. C. White, S. Lyman, et al, *Science* 247, 1219 (1990); A. Vecchi, C. Garlanda, M. G. Lampugnani, M. Resnati, C. Matteucci, et al, *Eur. J. Cell Biol.* 63, 247 (1994); T. N. Sato, Y. Tozawa, U. Deutsch, K. Wolburg-Buchholz, Y. Fujiwara, et al, *Nature* 376, 70 (1995); H. Schnurch, W. Risau, *Development* 119, 957 (1993); M. P. Bevilacqua, *Annu. Rev. Immuno.* 11, 767 (1993).
- (15) S. P. Janssens, A. Shimouchi, T. Quertermous, D. B. Block, K. D. Bloch, *J. Biol. Chem.* 267, 14519 (1992); S. Lamos, P. A. Marsden, G. K. Li, P. Tempst, T. Michel, *Proc. Natl. Acad. Sci. USA* 89, 6348 (1992).
- (16) R. van der Zee, F. Zoliman, J. Passeri, C. Lekutat, M. Silver, et al, *Circulation*, in press.

- (17) K. Shibuki, D. Okada, *Nature* 358, 676 (1991).
 (18) A. S. Weyrich, X.-I. Ma, M. Buerke, T. Murohara, V. E. Armstead, et al, *Circ. Res.* 75, 692 (1994).

ciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGACATTTT CGGGCTCAGC CTGCGCACCC

30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGGGTAGGC ACTTTAGTAG TTCTCCTAAC

30

(19) B. Torok-Storb, *Blood* 72, 373 (1988); N. Dainiak, *Blood* 78, 264 (1991).

(20) J. Folkman, M. Klagsbrun, *Science* 235, 442 (1987); R. O. Hynes, *Cell* 48, 549 (1987); P. C. Brooks, R. A. F. Clark, D. A. Cheresh, *Science* 264, 569 (1994); M. Friedlander, P. C. Brooks, R. W. Shaffer, C. M. Kincaid, J. A. Varner, et al, *Science* 270, 1500 (1995).

(21) P. J. Simmons, B. Masinovsky, B. M. Longenecker, R. Berenson, B. Torok-Storb, et al, *Blood* 80, 388 (1992); J. L. Liesveld, K. E. Frediani, A. W. Harbol, J. F. DiPersio, C. N. Abboud, *Leukemia* 8, 2111 (1994). (23) S. Takeshita, L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, et al, *J. Clin. Invest.* 93, 662 (1994); L. Q. Pu, A. D. Sniderman, R. Brassard, K. J. Lachapelle, A. M. Graham, et al, *Circulation* 88, 208 (1993); R. Baffour, J. Berman, J. L. Garb, S. W. Rhee, J. Kaufman, et al, *J. Vasc. Surg.* 16, 181 (1992).

(24) J. M. Isner, A. Pieczek, R. Schainfeld, R. Blair, L. Haley, et al, *Lancet* 348, 370 (1996).

(25) P. A. D'Amore, R. W. Thompson, *Annu. Rev. Physiol.* 49, 453 (1987).

(26) M. R. Tschudi, M. Barton, N. A. Bersinger, P. Moreau, F. Cosentino, et al, *J. Clin. Invest* 98, 899 (1996).

(27) Y. Sato, K. Okamura, A. Morimoto, R. Hamanaka, K. Hamanaguchi, et al, *Exp. Cell Res.* 204, 223 (1993); M. S. Pepper, N. Ferrara, L. Orci, R. Montesano, *Biochem Biophys Res Comm* 181, 902 (1991); D. R. Senger, S. R. Ledbetter, K. P. Claffey, A. Papadopoulos-Sergiou, C. A. Perruzzi, et al, *Am. J. Pathol.* 149, 293 (1996).

This invention has been described in detail including the preferred embodiments thereof. However, it will be appre-

What is claimed is:

1. A method for inducing the formation of new blood vessels in an ischemic tissue in a patient in need thereof, comprising:

administering to said patient host an effective amount of an isolated endothelial progenitor cell to induce new blood vessel formation in said ischemic tissue, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺.

2. The method of claim 1, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

3. The method of claim 2, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β , platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α , hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

4. The method of claim 3, wherein the endothelial cell mitogen is vascular endothelial growth factor.

5. The method of claim 1, wherein said patient is in need of treatment for cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

6. A method of enhancing blood vessel formation in a patient in need thereof, comprising:

a. selecting the patient in need thereof;

b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺; and

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- c. readministering the endothelial progenitor cells to the patient.
- 7. A method for treating an injured blood vessel in a patient in need thereof, comprising:
 - a. selecting the patient in need thereof; and
 - b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺; and
 - c. readministering the endothelial progenitor cells to the patient.
- 8. The method of claim 7, wherein the injury is the result of balloon angioplasty.
- 9. The method of claim 7, wherein the injury is the result of deployment of an endovascular stent.

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10. The method of claim 7, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

- 11. The method of claim 10, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β , platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α , hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxidesynthase.

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EVIDENCE APPENDIX

ITEM NO. 2

**Asahara, et al., 1997 Science article entitled,
“Isolation of Putative Progenitor Endothelial Cells for Angiogenesis”
cited by Appellant as Exhibit A in Appeal Brief filed herewith**

Isolation of Putative Progenitor Endothelial Cells for Angiogenesis

Takayuki Asahara, Toyooki Murohara, Alison Sullivan, Marcy Silver, Rien van der Zee, Tong Li, Bernhard Witzenbichler, Gina Schatteman, Jeffrey M. Isner*

Putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and for delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis.

Postnatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to the formation of embryonic blood vessels from EC progenitors, or angioblasts (2).

Vasculogenesis begins as a cluster formation, or blood island, comprising angioblasts at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this spatial association, angioblasts and HSCs share certain antigenic determinants, including Flk-1, Tie-2, and CD34. Conceivably, then, these progenitor cells may derive from a common precursor (3, 4).

The demonstration that HSCs from peripheral blood can provide sustained hematopoietic recovery is inferential evidence for circulating stem cells (5). Here, we have investigated the hypothesis that peripheral blood contains cells that can differentiate into ECs (6). We exploited two antigens that are shared by angioblasts and HSCs to isolate putative angioblasts from the leukocyte fraction of peripheral blood. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (7). It is also expressed by many including most activated ECs in the adult (8). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (9), is also expressed by both early HSCs and ECs but ceases to be expressed during hematopoietic differentiation (10, 11).

CD34-positive mononuclear blood cells (MB^{CD34+}) were isolated from human peripheral blood by means of magnetic beads

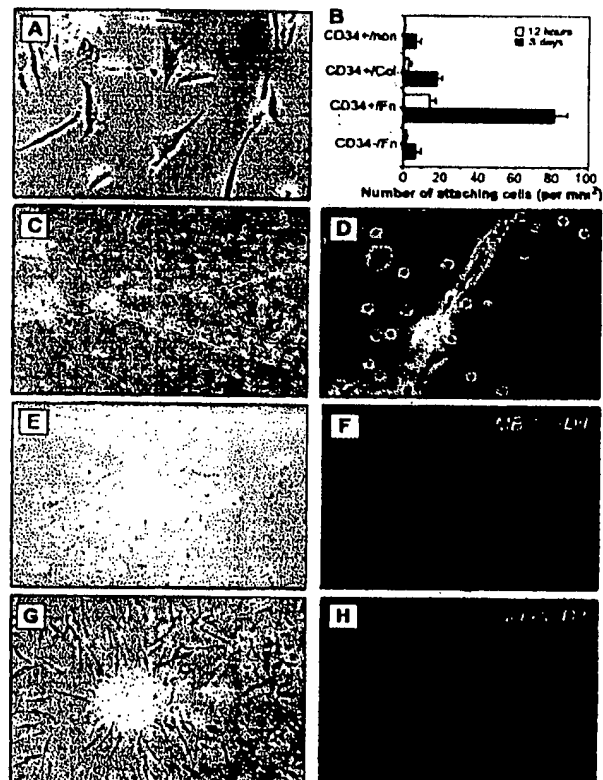
coated with antibody to CD34 (Dyna, Lake Success) (12). Fluorescence-activated cell sorting (FACS) analysis (13) indicated that $15.7 \pm 3.3\%$ of selected cells compared with $<0.1\%$ of the remaining cells expressed CD34. CD34-depleted cells (MB^{CD34-}) were used as controls. An antibody to Flk-1 was used for magnetic bead selection of Flk-1-positive mononuclear

blood cells (MB^{Flk1+}); among MB^{Flk1+} cells, $20.0 \pm 3.3\%$ were Flk-1 positive.

The MB^{CD34+} and MB^{CD34-} cells were plated separately (14) on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of 1×10^3 cells/mm², a limited number of MB^{CD34+} attached, became spindle shaped, and proliferated for 4 weeks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (Fig. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (probability $P < 0.05$, by analysis of variance) (Fig. 1B). Attached cells were observed only sporadically among MB^{CD34-} cultures, including cells followed for up to 4 weeks on fibronectin-coated plates.

To confirm that the spindle-shaped cells were derived from CD34-positive cells, we labeled MB^{CD34+} cells with the fluorescent dye Dil and cocultured them with unlabeled MB^{CD34-} cells on fibronectin at an overall density of 5×10^3 cells/mm²; the ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^{CD34+} , 99% MB^{CD34-}). After 7 days, Dil-labeled cells derived from the MB^{CD34+} culture, which initially account-

Fig. 1. Attachment, cluster formation, and capillary network development by progenitor ECs in vitro. (A) Spindle-shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} (50 cells/mm²) on fibronectin in standard medium (14). (B) Number of AT^{CD34+} cells 12 hours and 3 days after culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/Col), or fibronectin (CD34+/Fn), and MB^{CD34-} on fibronectin (CD34-/Fn). Network formation (C) and cord-like structures (D) were observed 48 hours after plating coculture of MB^{CD34+} , labeled with Dil, with unlabeled MB^{CD34-} cells (ratio of 1:100) on fibronectin. At 12 hours after coculture, MB^{CD34+} -derived cells had formed multiple clusters (E and F). After 5 days, uptake of acLDL-Dil was detected in AT^{CD34+} cells at the periphery but not the center of the cluster (G and H).



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ed for only 1% of the blood cells, accounted for $60.3 \pm 4.7\%$ of total attaching cells as analyzed by FACS. Coincubation with MB^{CD34+} cells increased the proliferation rate to more than 10 times that of MB^{CD34+} plated alone. Cocultures of MB^{CD34+} and MB^{CD34+} cells also showed enhanced MB^{CD34+} differentiation, including the formation of cellular networks and tube-like structures on fibronectin-coated plates (Fig. 1, C and D). These structures consisted principally of Dil-labeled MB^{CD34+} -derived cells (Fig. 1D). Furthermore, within 12 hours of coculture, multiple clusters had formed (Fig. 1E) that contained mostly MB^{CD34+} -derived cells (Fig. 1F). These clusters comprised round cells centrally and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to ECs and vascular structures *in vitro* (3). AT^{CD34+} cells at the cluster periphery took up Dil-labeled acetylated low density lipoprotein (acLDL), whereas the round cells did not (Fig. 1, G and H); the latter detached from the cluster several days later. The MB^{Flk-1+} cells behaved similarly.

To evaluate whether MB^{CD34+} cells progressed to an EC-like phenotype, we assayed them for the expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} cells, AT^{CD34+} cells cultured on fibronectin for 7 days, and human umbilical vein endothelial cells (HUVECs) were incubated with fluorescent-labeled antibodies and analyzed by FACS (Fig. 2). Leukocyte common antigen CD45 was identified on 94.1% of freshly

isolated cells but disappeared after 7 days of culture (Fig. 2). In freshly isolated MB^{CD34+} cells, $15.7 \pm 3.3\%$ were $CD34^+$, $27.6 \pm 4.3\%$ were $Flk-1^+$, and $10.8 \pm 0.9\%$ were $CD34^+Flk-1^+$. Expression of $CD34$, $CD31$, $Flk-1$, $Tie-2$, and E selectin—all markers of the EC lineage (11, 15)—was greater in AT^{CD34+} cells after 7 days of culture than in freshly isolated MB^{CD34+} cells.

Additional analyses (16) of AT^{CD34+} cells after 7 days of culture showed limited ($6.0 \pm 2.4\%$ cells) expression of CD68, a marker of the monocyte-macrophage lineage; positive immunostaining for factor VIII, ulex europaeus agglutinin-1 (UEA-1), CD31, endothelial constitutive nitric oxide synthase (ecNOS), and E selectin; and more than 80% uptake of Dil-labeled acLDL.

To confirm an EC-like phenotype of AT^{CD34+} cells, we documented expression of ecNOS, $Flk-1/KDR$ ($Flk-1$ is also known as VEGFR-2 in mouse, and KDR is the human homolog of VEGFR-2), and $CD31$ mRNA at 7, 14, and 21 days by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Evidence for ecNOS and $Flk-1/KDR$ in AT^{CD34+} cells was also demonstrated in a functional assay. Nitric oxide was produced in the cells in response to the EC-dependent agonist acetylcholine (ACh) and the EC-specific mitogen VEGF (Fig. 3B); the latter response also confirms that the cells express a functional $Flk-1$ receptor (17).

To determine if MB^{CD34+} cells contribute to angiogenesis *in vivo*, we used mouse and rabbit models of hindlimb ischemia. For administration of human MB^{CD34+} cells, C57BL/6J \times 129/SV background athymic

nude mice were used to avoid potential graft-versus-host complications. Two days after creating unilateral hindlimb ischemia by excising one femoral artery, we injected mice with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34+} cells into the tail vein. Histologic examination 1 to 6 weeks later revealed numerous (Fig. 4A) including proliferative (Fig. 4, C and D) Dil-labeled cells in the neovascularized ischemic hindlimb. Nearly all labeled cells appeared integrated into capillary vessel walls. In MB^{CD34+} -injected mice, $13.4 \pm 5.7\%$ of all $CD31$ -positive capillaries contained Dil-labeled cells, compared with $1.6 \pm 0.8\%$ in MB^{CD34+} -injected mice (18). By 6 weeks, Dil-labeled cells were clearly arranged into capillaries among preserved muscle structures (Fig. 4, I and J).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34+} -injected mice. Dil-labeled cells consistently colocalized with cells immunostained for $CD31$ (Fig. 4, B, F, and J), $Tie-2$ (Fig. 4G), and UEA-1 lectin (16). In contrast, in hindlimb sections from mice injected with MB^{CD34+} , Dil-labeled cells were typically found in stroma near capillaries, but they did not form part of the vessel wall nor did they colocalize with cells that stained with antibodies to either UEA-1 or $CD31$ (Fig. 4, K and L).

In a second set of mouse experiments, 1×10^4 MB^{Flk-1+} cells were isolated from

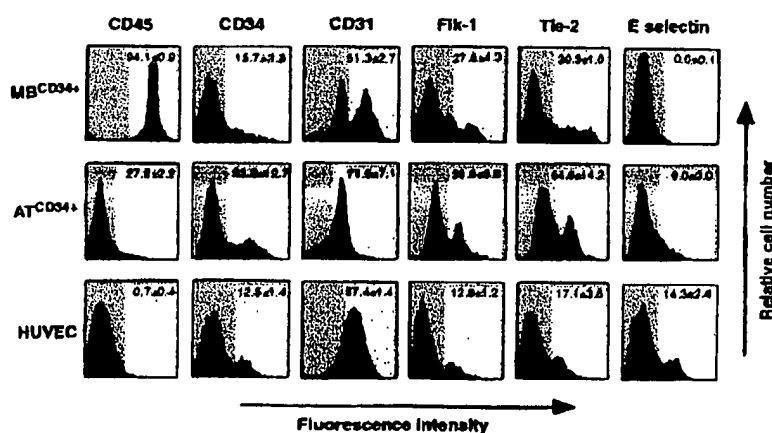


Fig. 2. FACS analysis of freshly isolated MB^{CD34+} and AT^{CD34+} cells after 7 days in culture, and HUVECs. Cells were labeled with fluorescent antibodies to CD45 (DAKO, Carpinteria); CD34, CD31 (Bioss); $Flk-1$, $Tie-2$ (Santa Cruz); and E selectin (DAKO). Similar results were obtained in three or more experiments. The shaded area of each box denotes negative antigen gate, and the white area denotes positive gate. Numbers are the mean \pm SEM percentage of cells for all experiments determined by comparison with corresponding negative control labeling.

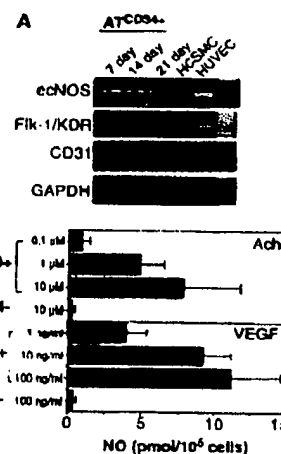


Fig. 3. Progenitor ECs express ecNOS, $Flk-1/KDR$, and $CD31$ mRNA and release NO. (A) Complementary DNA (from 10^6 cells) was amplified by PCR (40 cycles) with paired primers (23). (B) NO release from AT^{CD34+} and AT^{CD34-} cells cultured in six-well plates was measured as described (24). NO production was measured in a well with incremental doses of VEGF and ACh. HUVECs and bovine aortic ECs were used as positive controls, and human coronary smooth muscle cells (HCSMCs) as negative control. The values are means \pm SEM of 10 measurements for each group.

whole blood of 10 transgenic mice constitutively overexpressing β -galactosidase (β -Gal) (all mice were Flk-1^{+/+}). MB^{Flk-1+} or MB^{Flk-1-} cells were injected into nontransgenic mice of the same genetic background that had hindlimb ischemia of 2 days duration. Immunostaining of ischemic tissue, harvested 4 weeks after injection, for β -Gal demonstrated incorporation of cells expressing β -Gal in capillaries and small ar-

teries (Fig. 4M); these cells were identified as ECs by staining with antibody to CD31 (anti-CD31) and BS-1 lectin.

In vivo incorporation of autologous MB^{CD34+} cells into foci of neovascularization was also tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} cells were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand White rabbits immediately

before surgical induction of unilateral hindlimb ischemia (19). Immediately after surgery, freshly isolated autologous Dil-labeled MB^{CD34+} were reinjected into the ear vein of the same rabbit. Histologic examination of the ischemic limbs 4 weeks later revealed that Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb (Fig. 4, N and O) and were incorporated into $9.7 \pm 4.5\%$ of the capillaries that

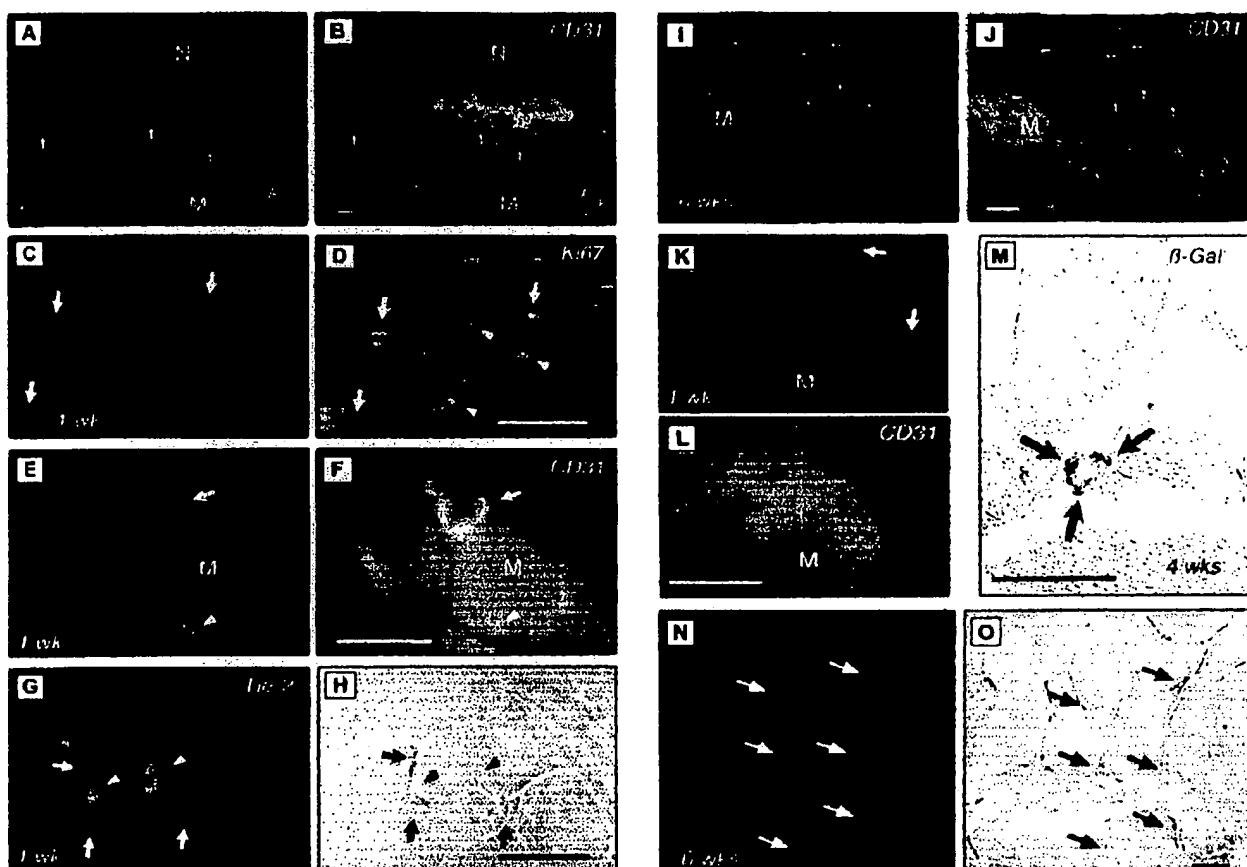


Fig. 4. Heterologous (panels A to L), homologous (M), or autologous (panels N and O) EC progenitors incorporate into sites of angiogenesis in vivo. (A and B) Dil-labeled MB^{CD34+} (red, arrows) between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are colabeled with CD31 (green, arrows). Note a preexisting artery (A), identified as CD31-positive, but Dil-negative. (C and D) Evidence of proliferative activity among several Dil-labeled MB^{CD34+}-derived cells (red, arrows), indicated by colmunostaining for antibody to Ki67 (Vector Lab, Burlingame, California) (green). Proliferative activity is also seen among Dil-negative, Ki67-positive capillary ECs (arrowheads); both cell types contribute to neovasculation. (E) Dil (red) and CD31 (green) in capillary ECs (arrows in E and F) between skeletal myocytes, photographed through a double filter 1 week after Dil-labeled MB^{CD34+} injection. (F) A single green filter shows CD31 (green) expression in Dil-labeled capillary ECs integrated into the capillary with native (Dil-negative, CD31-positive) ECs (arrowheads in E and F). (G) Immunostaining 1 week after MB^{CD34+} injection showing capillaries comprising Dil-labeled MB^{CD34+}-derived cells expressing Tie-2 receptor (green). Several MB^{CD34+}-derived cells (arrows) Tie-2 positive and integrated with some Tie-2-positive

host capillary cells (arrowheads) identified by the absence of red fluorescence. (H) Phase-contrast photomicrograph of the same section shown in (G) indicates the corresponding Dil-labeled (arrows) and -unlabeled (arrowheads) capillary ECs. (I and J) Six weeks after administration, MB^{CD34+}-derived cells (red, arrows) colabel for CD31 in capillaries between preserved skeletal myocytes (M). (K and L) One week after injection of MB^{CD34+}, isolated MB^{CD34+}-derived cells (red, arrows) are observed between myocytes but do not express CD31. (M) Immunostaining of β -Gal in a tissue section harvested from ischemic muscle of C57BL/6J, 129/SV mice 4 weeks after the administration of MB^{Flk-1+} isolated from transgenic mice constitutively expressing β -Gal. (Flk-1 cell isolation was used for selection of EC progenitors because of the lack of a suitable antibody to mouse CD34.) Cells overexpressing β -Gal (arrows) were incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and BS-1 lectin (16). (N and O) Section of muscle harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous MB^{CD34+} cells. Red fluorescence in (N) indicates localization of MB^{CD34+}-derived cells in capillaries seen (arrows) in the phase-contrast photomicrograph in (O). Each scale bar is 50 μ m.

consistently expressed CD31 and reacted with DS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti-Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk-1+} cells may contribute to neovascularization in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2, 3). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (20) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (21). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For antineoplastic therapies, MB^{CD34+} cells could be transduced with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (22).

REFERENCES AND NOTES

1. J. Folkman and Y. Shing, *J. Biol. Chem.* **267**, 10931 (1992); W. Schaper, M. D. Brahander, P. Low, *Circ. Res.* **28**, 671 (1971); W. Risau, *FASEB J.* **9**, 926 (1995).
2. W. Risau et al., *Development* **102**, 471 (1988); L. Perleaud, C. Altman, P. Kito, F. Dieterlen-Lièvre, *ibid.* **105**, 473 (1989).
3. I. Flamme and W. Risau, *ibid.* **116**, 435 (1992).
4. W. Hs, Abhandl. K.S. Ges. Wiss. Math.-Phys. **22**, 171 (1900); M. Weiss and S. H. Orkin, *J. Clin. Invest.* **97**, 591 (1996).
5. W. Brugger, S. Helmfield, R. J. Berenson, R. Montelmann, L. Kan, *N. Engl. J. Med.* **333**, 283 (1995).
6. M. Wu et al., *J. Vasc. Surg.* **21**, 862 (1995); Q. Shi et al., *ibid.* **20**, 546 (1994); Y. Nishiki, Y. Tomizawa, Y. Yamano, A. Matsunaga, *Nature Med.* **2**, 90 (1996).
7. C. I. Chin et al., *J. Immunol.* **133**, 157 (1984); F. Katz, R. W. Tindle, D. H. Sutherland, M. D. Greaves, *Leukemia Res.* **9**, 191 (1985); R. G. Andrews, J. W. Singer, I. D. Bernstein, *Blood* **67**, 842 (1986).
8. J. Fina et al., *Blood* **75**, 2417 (1990); D. Soligo et al., *Leukemia (Wusinstok)* **5**, 1026 (1991); A. Ito, S. Nomura, S. Hirota, I. Suda, Y. Kitamura, *Lab. Invest.* **72**, 532 (1995).
9. C. deVries et al., *Science* **255**, 989 (1992); B. I. Terman et al., *Biochem. Biophys. Res. Commun.* **187**, 1579 (1992); F. Shalaby et al., *Nature* **376**, 62 (1995).
10. W. Matthews et al., *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9026 (1991).
11. B. Millauer et al., *Cell* **72**, 835 (1993); T. P. Yamaguchi, D. J. Dumont, R. A. Conlon, M. L. Brallman, J. Rossant, *Development* **118**, 489 (1993).
12. Single donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
13. MB^{CD34+}, MB^{CD34-}, and MB^{Flk-1+} cells (>1 × 10⁶ of each) were analyzed with anti-CD34 (Biossigen, Kennebunkport, ME) and anti-Flk-1 (Santa Cruz Biotechnologies, Santa Cruz, CA).
14. The medium for all cell culture experiments was M-199 with 20% fetal bovine serum and bovine brain

extract (Clonetics, San Diego).

15. P. J. Newman et al., *Science* **247**, 1219 (1990); T. N. Satoh et al., *Nature* **376**, 70 (1995); H. Schnurch and W. Risau, *Development* **119**, 957 (1993); M. P. Bevilacqua, *Arter. Rev. Immunol.* **11**, 767 (1993).
16. T. Asashara et al., data not shown.
17. H. van der Zee et al., *Circulation*, in press.
18. The mean percent of DiI-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields (×400).
19. New Zealand White rabbits (3.8 to 4.2 kg, n = 4, Pine Acre Rabbits, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised [S. Takeshita et al., *J. Clin. Invest.* **93**, 662 (1994); L. O. Fu et al., *Circulation* **88**, 208 (1993); R. Baffour et al., *J. Vasc. Surg.* **16**, 181 (1992)].
20. J. M. Isner et al., *Lancet* **348**, 370 (1996).
21. M. R. Tedeschi et al., *J. Clin. Invest.* **98**, 699 (1996).
22. Y. Sato et al., *Exp. Cell Res.* **204**, 223 (1993); M. S. Pepper, N. Ferrara, L. Orci, R. Montesano, *Biochem. Biophys. Res. Commun.* **181**, 902 (1991); D. R. Senger et al., *Am. J. Pathol.* **149**, 283 (1996).
23. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for β-actin, AAG ACA TTT TGG GGC TCA CGC TGC GCA CCC/ TGG GGT AGC CAC TTT AGT AGT TCT CCT AAC (548-base pairs (bp) PCR product); for Flk-1 (KDR), CAA CAA AGT CGG GAG AGG AG/ATG ACG A1G GAC AAG TAG CC (819-bp PCR product); for CD31, GCT G1 T GGT GGA AGC AGT GC/GAA GTT GGC TGG AGG TGC TC (645-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/ CAT GTG GGC CAT GAG GTC CAC CAC (1983-bp PCR product).
24. NO release was measured with a NO-specific polarographic electrode connected to a NO meter (iso-NO, World Precision Instruments, Sarasota, FL). ATC³⁰⁴ or ATC³⁰⁴ cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.
25. Supported by grants from NIH National Heart, Lung, and Blood Institute numbers 02824, 53354, and 57516, the American Heart Association, the E. L. Wiegand Foundation, and in part by the Uehara Memorial Foundation (T.M.).

4 October 1995; accepted 14 January 1997

Somatic Frameshift Mutations in the BAX Gene in Colon Cancers of the Microsatellite Mutator Phenotype

Nicholas Rampino, Hiroyuki Yamamoto, Yuriy Ionov, Yan Li, Hisako Sawai, John C. Reed, Manuel Perucho*

Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP⁺ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [(G)₈] within BAX, a gene that promotes apoptosis. These mutations were absent in MMP⁻ tumors and were significantly less frequent in (G)_n repeats from other genes. Frameshift mutations were present in both BAX alleles in some MMP⁺ colon tumor cell lines and in primary tumors. These results suggest that inactivating BAX mutations are selected for during the progression of colorectal MMP⁺ tumors and that the wild-type BAX gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (1-3). The fixation of these slip-py-induced replication errors as mutations (4) is associated with defects in DNA mismatch repair (5). Colorectal MMP⁺ tumors frequently contain frameshift mutations in the type II transforming growth factor-β (TGF-β) receptor gene (6) but are usually wild type for the p53 tumor suppressor gene (1, 7). In addition to its central role in cell growth arrest (8), p53 also plays a role in apoptosis in response to DNA

damage (9). The p53 protein transactivates BAX (10), a member of the BCL2 gene family (11) that promotes apoptosis (12).

The human BAX gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (12). To determine whether this sequence is a mutational target in MMP⁺ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the (G)₈ tract from various MMP⁺ tumor cell lines. This analysis revealed hand shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a BAX fragment with mutant (G)_n and (C)_n tracts (Fig. 1A, P9 and P7).

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EVIDENCE APPENDIX

ITEM NO. 3

Declaration of Dr. Richard Heuser filed November 22, 2004.



PATENT
Appl. No. 09/064,000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia
Serial No.: 09/064,000
Filed: April 21, 1998
For: METHOD AND APPARATUS
FOR INSTALLATION OF
DENTAL IMPLANT

Group Art Unit: 1646

Examiner: Elizabeth Kemmerer, Ph.D.

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class mail, in an envelope addressed to MAIL STOP AF, Commissioner for Patents, P.O. Box 1450, Arlington, VA 22313-1450 on:

NOVEMBER 16, 2004

Gerald K. White 11/16/04
Signature Date of signature

LETTER

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

Sir:

Enclosed herewith, please find the Declaration of Richard Heuser, M.D.

This Declaration is being submitted in an effort to reduce the number of issues in the instant application and thereby expedite the prosecution thereof.

Respectfully submitted,

Date: November 16, 2004

Gerald K. White

Gerald K. White
Reg. No. 26,611

GERALD K. WHITE & ASSOCIATES, P.C.
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

DECLARATION OF RICHARD HEUSER, M.D.

I Richard Heuser declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.
2. My Curriculum Vitae ("CV") is attached hereto as Exhibit A.
3. In addition to my CV, I am currently Director of Cardiovascular Research at St. Joseph's Hospital and Medicine Center, and I serve as Clinical Professor of Medicine at University of Arizona College of Medicine. Over the past six years, I have worked in gene therapy, as well as muscle regeneration for the treatment of cardiomyopathy.

In my CV, you will note reference to work that was done with Sulzer Medical involving a rabbit hind limb model to stimulate peripheral vascular disease. I injected a growth mixture that included FGF, etc. into the hind limb model.

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Exhibit B.
5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
6. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C. Also included in Exhibit C is a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary, a service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions

are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

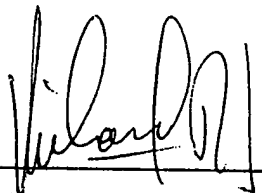
7. I have read and understood the claims set forth in Exhibit D and have been informed that such claims are present in the above-referenced patent application. It is my opinion that those skilled in the medical arts, reading such claims would understand that cells including stem cells, are species of living organisms.
8. The publication in attached Exhibit E illustrates that placement of a growth factor, including cells, and more specifically, stem cells, in a human patient forms soft tissue, such as an artery. This publication reports work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.
9. Based upon above Paragraphs 4-8, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery.
10. Based upon above Paragraphs 4-7, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.
11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

11/11/04

A handwritten signature in black ink, appearing to read "Richard Heuser", is written over a horizontal line.

Richard Heuser

EXHIBIT A

CURRICULUM VITAE

Curriculum Vitae
Richard Ross Heuser, M.D., F.A.C.C., F.A.C.P.

ADDRESS:

525 North 18th Street, Suite 504
Phoenix, Arizona 85006
(602) 234-0004
(602) 234-0058 (fax)
phoenixheart@earthlink.net

EDUCATION:

1969 - 1972

University of Wisconsin
Honors in Chemistry
Phi Beta Kappa
Evan Hefner Scholarship in Chemistry

1972 - 1976

University of Wisconsin School of Medicine
Graduation with Honors - May 1976
Alpha Omega Alpha
Evan Hefner Scholarship in Medicine

POST GRADUATE TRAINING:

1976 - 1977

Internship in Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

1977 - 1979

Residency in Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

1979 - 1981

Fellowship in Cardiology
The Johns Hopkins Hospital
Baltimore, Maryland

LICENSURE:

State of Arizona, License #19703
State of New Mexico, License #83-220

EMPLOYMENT:

December 2002 - Present

Director of Cardiovascular Research
St. Joseph's Hospital and Medical Center
Phoenix, Arizona

April 2001 - Present

Cardiac Cath Lab Director
St. Luke's Medical Center, Phoenix, Arizona

June 2000 - Present

Medical Director
Discovery Alliance, Phoenix, Arizona

1998 - June 2000

Director
Phoenix Research Center, Phoenix, Arizona

April 1997 - Present	Medical Director Phoenix Heart Center, Phoenix, Arizona
December 1999 - Present	Director of Research St. Luke's Medical Center, Phoenix, Arizona
April 1997 - December 1999	Director of Research and Education Phoenix Regional Medical Center, Phoenix, Arizona
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute, Phoenix, Arizona
July 1983 - April 1990	Private Practice New Mexico Heart Clinic, Albuquerque, New Mexico
July 1982 - June 1983	Private Practice Houston Cardiovascular Associates, Houston, Texas
June 1981 - July 1982	Instructor in Medicine, Cardiology The Johns Hopkins Hospital, Baltimore, Maryland

PROFESSIONAL APPOINTMENTS:

1981 - July 1982	Instructor in Medicine - Cardiology Division of Cardiology The Johns Hopkins Hospital, Baltimore, Maryland
July 1982 - June 1983	Instructor in Medicine, Cardiology Baylor College of Medicine, Houston, Texas
July 1983 - February 1990	Director, Interventional Cardiology New Mexico Heart Clinic, Albuquerque, New Mexico
April 1984 - June 1986	Clinical Assistant Professor of Medicine University of New Mexico, Albuquerque, New Mexico Director, Medical Residency Program New Mexico Heart Clinic, Albuquerque, New Mexico
June 1986 - April 1990	Clinical Associate Professor of Medicine University of New Mexico, Albuquerque, New Mexico
May 1996 - April 1997	Director, Interventional Cardiology Arizona Heart Institute Foundation, Phoenix, Arizona
Sept 1995 - December 1999	Medical Director - Cardiac Catheterization Laboratory Phoenix Regional Medical Center, Phoenix, Arizona
December 1990 - Present	Clinical Associate Professor of Medicine University of Louisville, Louisville, Kentucky
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute Foundation, Phoenix, Arizona

April 1997 - December 1999 Director of Research and Education
Phoenix Regional Medical Center, Phoenix, Arizona

BOARD MEMBERSHIPS:

American Board of Internal Medicine
American Board of Cardiovascular Diseases, Diplomat
American Board of Interventional Cardiovascular Diseases, Diplomat

PROFESSIONAL MEMBERSHIPS:

Fellow, American College of Angiology
Fellow, American College of Cardiology
Fellow, American College of Physicians
Fellow, of the American Heart Association
Fellow, American Society of Cardiovascular Interventions
Fellow, International Society of Cardiovascular Interventions
Fellow, Society for Cardiac Angiography and Interventions
Member, American Association for the Advancement of Science
Member, American Heart Association
Member, American Medical Association
Member, Houston Cardiology Society
Member, Houston Society of Internal Medicine
Member, International Andreas Grüntzig Society
Member, International Network of Interventional Cardiology
Member, International Society for Carotid Artery Therapy
Member, International Society for Minimally Invasive Cardiac Surgery
Member, New Mexico Medical Society
Member, Harris County Medical Society
Member, Texas Medical Association
Member, National Register's Who's Who in Executives and Professionals
Member, Who's Who in Medicine and Healthcare 2002-2003

CLINICAL ADVISORY BOARDS:

Advanced Cardiovascular Systems
USCI
Mansfield Scientific Interventional Board
Medtronic Interventional Vascular
Scientific Advisory Board of International Society of Heart Failure

EDITORIAL BOARDS:

Catheterization and Cardiovascular Diagnosis
Journal of Endovascular Surgery
Cardiovascular Research Foundation/Society of Cardiac Angiography and Interventions
Abstract Grader TCT

DATA SAFETY BOARDS:

- ICEM Data Safety Monitoring Board

- Abbott Laboratories Data Safety Monitoring Board for Drug Coated Stent Program, PREFER, A Perspective STUDY to Evaluate the Safety and Efficacy of the ABT-578 coated BiodivYsio® Stent for the Reduction of Restenosis

CONSULTANT TO:

Editors of the *Annals of Internal Medicine*
 Editors of *Catheterization and Cardiovascular Diagnosis*
 Editors of *Circulation*
 Editors of the *Journal of Invasive Cardiology*
 Editors of the *American Journal of Cardiology*
 Editors of *Web M.D.*
 Annual Scientific Session Program Committee of the American College of Cardiology
 Annual Scientific Session Program Committee of the American College of Cardiology
 Abstract Advisor for Angioplasty; Stents
 Annual International Symposium of Transcatheter Cardiovascular Therapeutics
 Abstract Grader

DEVICE RESEARCH:

Sub-Investigator	ACS Multi-Link Stent Trial Principal Investigator - ACS RX
Principal Investigator	ACT-One Trial Principal Investigator - Angio-Seal Trial
Principal Investigator	Balloon Expandable Intraluminal Stent for Subtotally Occluded Iliac Arteries
Principal Investigator	Bard® Memotherm Carotid Stent Study
Principal Investigator	BARRICADE Trial - The Barrier Approach to Restenosis: Restrict Intima and Curtail Adverse Events (JOMED JOSTENT)
Principal Investigator	BEST Trial
Principal Investigator	BetaCath System Trial
Principal Investigator	Boehringer Ingelheim Pharmaceuticals Protocol Comparing Micardis and COZAAR
Principal Investigator	CABERNET Clinical Trial - Carotid Artery Revascularization using the Boston Scientific EPI FiltreWire EX™ and the EndoTex™ NexStent™
Principal Investigator	CADILLAC Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	CAPTIVE - Cardioshield Application Protects During Transluminal Intervention of Vein Grafts by Reducing Emboli
Principal Investigator	CARDIOMETRICS
Principal Investigator	Carotid Wallstent Trial
Principal Investigator	CAVEAT II Trial
Principal Investigator	Clinical Investigation of the Magnum Wire vs. Standard Guide Wires during Total Occlusion Angioplasty
Principal Investigator	Cook GR II Trial
Principal Investigator	CORDIS Nitinol Carotid Stent And Delivery System for the Treatment of Obstructive Carotid Artery Disease
Principal Investigator	Cordis Carotid Randomized Sapphire
Principal Investigator	Cordis Bilateral AAA Device & Delivery System
Principal Investigator	(CATS) Safe-Steer™ Wire System Coronary Artery Total Occlusion Study
Principal Investigator	CREDO Trial
Principal Investigator	Novoste CUP Trial
Principal Investigator	CVD Accucath Infusion Catheter
Principal Investigator	Duett Closure Device
Principal Investigator	EndoSonics Cath scanner Oracle - PTCA Catheter

Principal Investigator EPI FilterWire EX™ System During Transluminal Intervention of Saphenous Vein Grafts

Principal Investigator Extra Stent

Principal Investigator GREAT - Guided Radio Frequency Energy Ablation of Total Occlusions Using the Safe Cross™ Radio Frequency Total Occlusion Crossing System

Principal Investigator GRIP - Guided Radio Frequency in Peripheral Total Occlusions using the Safe-Cross™ Radio Frequency (RF) Total Occlusion (TO) Crossing System

Principal Investigator HIPS Trial

Principal Investigator Human Percutaneous Laser Angioplasty of the Coronary Arteries

Principal Investigator Johnson & Johnson Intracoronary Stent Program Supplement #27 "New" Delivery System

Principal Investigator Kensey Nash Hemostatic Puncture Closure Device

Principal Investigator Mansfield-Boston Scientific Strecker Coronary Stent

Principal Investigator Medtronic AVE S7 with Discrete Technology Coronary Stent System

Principal Investigator Medtronic AVE S7 Coronary Stent Registry

Principal Investigator MOBILE Trial - More Patency with Beta for In-Stent Restenosis in the Lower Extremities Trial IDE #G010295; Protocol D00789 Rev B dated 12/01

Principal Investigator NIR Stent Trial

Principal Investigator Neurex/Elan Pharmaceuticals Trial

Principal Investigator PAMI Stent Trial

Principal Investigator Paragon Stent

Principal Investigator Paris Radiation Trial

Principal Investigator PaS Trial

Principal Investigator Percutaneous Coronary Angioscopy in Unstable Angina

Principal Investigator Percutaneous Recanalization of Stenotic Human Coronary Arteries with Balloon Expandable Intracoronary Stents

Principal Investigator Percutaneous Recanalization of Stenotic Human Saphenous Vein Bypass Graft with Balloon Expandable Intraluminal Stents

Principal Investigator Percutaneous Thermal Balloon Angioplasty

Principal Investigator PMR Trial

Principal Investigator Pravastatin or Atorvastatin Evaluation and Infection Therapy (Prove It)

Principal Investigator Presto Trial

Principal Investigator RAVES Trial

Principal Investigator RESCUE Trial

Principal Investigator SAFER - Saphenous Vein Graft Angioplasty Free of Emboli Randomized Study Using the PercuSurge Guard Wire™ System

Principal Investigator SAVED Trial

Principal Investigator Schering-Plough Phase III Study of SCH 58235 in addition to Pravastatin compared to placebo in subjects with primary hypercholesterolemia

Principal Investigator Long-Term, Open-Label, Safety and Tolerability Study of SCH 58235 in Addition to Pravastatin in Patients with Primary Hypercholesterolemia

Principal Investigator Schneider WINS Trial

Principal Investigator SCORES Trial

Principal Investigator Sepracor Study of Norastemizole in Cardiac Compromised Subjects

Principal Investigator SMART Trial (National PI)

Principal Investigator SMART: Post-Approval Study

Principal Investigator SNAPIST - A Phase 2, Safety Study of Systemic Nanoparticle Paclitaxel (ABI-007) For In-Stent Restenosis; IND #63,082

Principal Investigator SOAR - Renal Stent

Principal Investigator Efficacy and Safety Study of the Oral Direct Thrombin Inhibitor H 376/95 Compared with Dose-Adjusted Warfarin (Coumadin) in the Prevention of Stroke and Systemic Embolic Events in Patients with Atrial Fibrillation (SPORTIF V)

Principal Investigator STARS Trial

Principal Investigator START Trial (National PI)

Principal Investigator STRATUS Trial

Principal Investigator STRESS III Trial

Principal Investigator	SUMO Trial
Principal Investigator	(SWING) Sound Wave Inhibition of Neointimal Growth
Principal Investigator	Talent Endoluminal Graft (High Risk & Low Risk)
Principal Investigator	Talent Endoluminal Spring Stent-Graft System
Principal Investigator	Tenax-XR Coronary Stent System
Principal Investigator	TITAN Trial
Principal Investigator	Trimedyn Excimer Laser Assisted Percutaneous Coronary Angioplasty
Sub-Investigator	Trimedyn Percutaneous Eclipse Holmium Laser Coronary Angioplasty
Principal Investigator	VeGAS 2 Trial
Principal Investigator	Velocity Trial Principal Investigator - Venus Stent
Co-Investigator	WALLSTENT Study
Principal Investigator	WIKTOR Coronary Stent

PHARMACOLOGY RESEARCH:

Principal Investigator	Abbott rUK Trial
Principal Investigator	Ajinimoto Pharmaceuticals Double-Blind Placebo-Controlled Study of AT-1015 in Patients with Intermittent Claudication due to peripheral arterial disease
Sub-Investigator	Amgen, Inc. Anakinra Trial for Rheumatoid Arthritis
Principal Investigator	Astra Zeneca Pharmaceutical Trial to Evaluate the Safety and Efficacy of XXXX and Atorvastatin
Principal Investigator	Astra Zeneca Trial Open Label Dose Comparison Study to Evaluate the Safety and Efficacy of Rosuvastatin versus Atorvastatin, Pravastatin, and Simvastatin in Subjects with Hypercholesterolemia
Principal Investigator	Parke-Davis and Pfizer Randomized Open-Label Study Comparing the Efficacy of Once Daily Atorvastatin to Simvastatin in Hypercholesterolemic Patients
Principal Investigator	Pilot Study to Evaluate Intracoronary Administration of Activase for the Treatment of Intracoronary Thrombus
Principal Investigator	Artistic Trial
Principal Investigator	AstraZeneca Trial of Niaspan versus New Generation Statin for the Treatment of Type IIB and Type IV Hyperlipidemia
Principal Investigator	AstraZeneca Multicenter Trial for drug (XXX) and Atorvastatin for the Treatment of Hypercholesterolemia
Principal Investigator	BRAVO Trial
Principal Investigator	BioVall Angina & Hypertension Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	Challenge Trial
Sub-Investigator	Comparison of Lopentol and Omnipaque in Adult Angiocardiology
Sub-Investigator	Comparison of Intravenous Adenosine to Intravenous Placebo in Termination of Spontaneous or Induced Paroxysmal Supraventricular Tachycardia
Principal Investigator	Centacor Chimeric 7E3 Fab
Principal Investigator	COR Therapeutics Randomized Placebo-Controlled Dose Ranging Study of drug (XXXX) in Patients with Atherosclerotic Cardiovascular, Peripheral Vascular, and/or Cerebrovascular Disease
Sub-Investigator	Dose Response Study of Bucindolol in Patients with Congestive Heart Failure
Principal Investigator	Effects of Recombinant Human Superoxide Dismutase in Patients with Acute Myocardial Infarction Subject to Coronary Artery Reperfusion
Sub-Investigator	Eli Lilly - Agitation/Alzheimer's Trial
Principal Investigator	EPILOG Trial
Principal Investigator	ERASER Trial
Principal Investigator	GUSTO Trial
Principal Investigator	A multi-center, randomized, double blind, placebo-and-active controlled Parallel Group Dose-ranging Study of the HMG CoA Reductase Inhibitor, BMS-423526, in the treatment of Hyperlipidemia

Principal Investigator Study Lovastatin XL with MEVACOR in patients with hypercholesterolemia
 Sub-Investigator Lovastatin Multi-Center Trial
 Principal Investigator Extended Trial of Lovastatin XL for the treatment of hypercholesterolemia
 Principal Investigator Multicenter Double-Blind Placebo controlled trial of drug (XXXX) in patients with Type 2 Diabetes and Congestive Heart Failure
 Principal Investigator Effect of LDL-Cholesterol Lowering Beyond Currently Recommended Minimum Targets on coronary heart disease (CHD) Recurrence in patients with Pre-Existing CHD
 Principal Investigator A Double-Blind, Multi-Center, Randomized, Placebo-Controlled, Parallel Group Dosing Study Evaluating the Effects of Nebivolol on Blood Pressure in Patients with Mild to moderate Hypertension, NEB 302
 Principal Investigator Parallel Group Extension Study to Determine the Safety and Efficacy of Long-Term Nebivolol Exposure in Patients with Mild to Moderate Hypertension NEB 306,
 Sub-Investigator NeoTherapeutics Alzheimer's Disease 2000
 Sub-Investigator NeoTherapeutics Alzheimer's Disease 2001
 Principal Investigator OCTAVE Trial
 Sub-Investigator OCTAVE Trial
 Principal Investigator Pfizer Phase II Multicenter, double-blind placebo controlled randomized parallel group dose ranging study of the safety of CP529,414 soft-gel capsules
 Principal Investigator PLAC Trial
 Principal Investigator Protocol 073 Trial
 Principal Investigator Knoll Pharmaceutical Double-Blind Randomized Clinical Trial of Slow Release Propafenone (Rythmol-SR®) in the Prevention of Symptomatic Recurrences of Atrial Fibrillation
 Principal Investigator PREVAIL - A Phase 2 Multicenter, Double-Blind Placebo-Controlled, Dose-Ranging Study to Evaluate the Safety and Efficacy of BO-653 in Prevention of Post-Angioplasty Restenosis in Stented Lesions
 Principal Investigator PROVE-IT TIMI 22 - Pravastatin or Atorvastatin Evaluation and Infection Therapy
 Principal Investigator PURSUIT Trial
 Principal Investigator QUIET Trial
 Principal Investigator RAFT Trial
 Principal Investigator REPLACE Randomized Evaluation in PCI Linking Angiomax to reduce Clinical Events
 Sub-Investigator Safety and Efficacy Study of Burroughs - Wellcome Tissue Plasminogen Activator in Patients with Acute Myocardial Infarction
 Principal Investigator A 6-week, open-label, dose-comparison study to evaluate the safety and Efficacy of Rosuvastatin versus Atorvastatin, Cerivastatin, pravastatin, and Simvastatin in subjects with hypercholesterolemia
 Principal Investigator A 48-week, open-label, non-comparative, Multicentre, Phase IIIb study to evaluate the efficacy and safety of the Lipid-Regulating agent Rosuvastatin in the treatment of subjects with Fredrickson Type IIa and Type IIb Dyslipidemia, Including Heterozygous Familial Hypercholesterolemia
 Principal Investigator SAGE Trial
 Sub Investigator Long Term Open Label Safety and Tolerability Study of SCH58235 in addition to Pravastatin in Patient With Primary Hypercholesterolemia
 Principal Investigator Phase III Double-Blind Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
 Principal Investigator Phase III Open Label Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
 Principal Investigator Sepracor Protocol Study of Norastemizole in Cardiac Compromised Subjects
 Principal Investigator SPORTIF V - Atrial Fibrillation Trial
 Principal Investigator SWORD Trial
 Principal Investigator Titration-to-Response Trial Comparing Micardis and COZAAR® in Patients with Mild-to-moderate Hypertension

Principal Investigator TNT Trial
Principal Investigator TREND Trial
Sub-Investigator VALDECOXIB Trial
Principal Investigator An Open-Label, Multinational, Multicentre, Extension Trial to Assess the
Long-Term Safety and Efficacy of ZD4522 in Subjects in the ZD4522 Clinical Trial Program

BASIC RESEARCH:

- 1990 - 1993 Systematic assessment of Medtronic balloons and guiding catheters in porcine and canine models. Sponsored by Medtronic, Inc.
- 1990 - 1993 Determination of radiopacity and torquability of Medtronic vascular catheters in porcine models. Sponsored by Medtronic, Inc.
- 1992 - 1996 Evaluation of Strecker stent in porcine and canine models.
Sponsored by Boston Scientific
- Evaluation of Wiktor stent and stent in porcine and canine models.
Sponsored by Medtronic, Inc.
- Evaluation of NIR stent in porcine models.
Sponsored by Cordis Corp.
- 1990 - 1994 Evaluation of Japan Crescent radiofrequency balloon in porcine model with emphasis on histopathology of heat-produced lesions. Abstract submitted at 1993 AHA Conference.
- 1993 Evaluation of radiofrequency wire for total coronary occlusions in porcine models: Determining energy limitations. Equipment subsequently licensed to Radius Medical.
- 1994 - 1997 Training courses for professionals (physicians, engineers, technicians) in techniques and strategies for placement of coronary stents. Five courses sponsored by Johnson & Johnson, Medtronic, Inc. and Cook, Inc.
- 1997 Efficacy of the Endotex Abdominal Aortic Aneurysm exclusion device in a porcine model gauging ability to exclude renal arteries, ease of placement and radiopacity. Sponsored by Endotex
- 1998 Use of percutaneous myocardial revascularization in a porcine model.
Sponsored by Cardiogenesis Corporation at Stanford University.
- 1998 - 1999 Utility of radiofrequency (RF) percutaneous myocardial revascularization in acute and chronic porcine model: Histopathology and angiogenesis related to use of RF alone and in combination with growth factor (VEGF). Results presented at Angiogenesis 1999, Washington, DC.
- 1999 Development and testing of embolic probe device in porcine model (patent pending). Performed at PRMC and separately at Columbia Presbyterian in New York.
- 1999 Evaluation of the Medtronic carotid and SVG stent in porcine carotid and saphenous vein graft lesions assessing ease of use and 30-day outcome.
Sponsored by Medtronic, Inc.
- 1999 Development and testing of Protector vascular embolic protection device in

porcine model at Mayo Clinic (device patent pending).

- 1999 Evaluation of ability of intramuscular growth factor to stimulate angiogenesis in rabbit hindlimb model at 30 and 60 days post-procedure. Sponsored by Sulzer Medical.
- 1999 Use of Vesseal device to close porcine peripheral artery tears (patent #6,159,197) Sponsored by Phoenix Heart Center.

PUBLICATIONS:

- Bayless TM, **Heuser RR**: Fulminant Colitis. Johns Hopkins Medical Journal 1979 May;144(5):168-172.
- Heuser RR**, Achuff SC, Brinker JA: Inadvertent division of an anomalous left anterior descending coronary artery during complete repair of tetralogy of fallot. American Heart Journal 1982 Mar;103(3):430-432.
- Fuchs RM, **Heuser RR**, Yin FC, Brinker JA: Limitations of pulmonary wedge V-waves in diagnosing mitral regurgitation. The American Journal of Cardiology 1982 Mar;49(4):849-854.
- Fuchs RM, Brin KP, Brinker JA, Guzman PA, **Heuser RR**, Yin FC: Augmentation of regional coronary blood flow by intraaortic balloon counterpulsation in patients with unstable angina. Circulation 1983 Jul;68(1):117-123.
- Alexander EL, Weiss JL, Firestein GS, **Heuser RR**, Leitl G, Wagner Jr HN, Brinker JA, Ciuffo AA, Becker LC: Reversible cold-induced abnormalities in myocardial perfusion and function in systemic sclerosis. Annals of Internal Medical 1986 Nov;105(5):661-668.
- Heuser RR**: Cardiogenic shock treated by PTCA. Cardiology 1987;4(6):64-66.
- Heuser RR**, Maddoux GL, Goss JE, Ramo BW, Raff GL, Shadoff N: Coronary angioplasty for acute mitral regurgitation due to myocardial infarction: A nonsurgical treatment preserving mitral valve integrity. Annals of Internal Medical 1987 Dec;107(6):852-855.
- Maddoux GL, Goss JE, Ramo BW, Raff GL, **Heuser RR**, Shadoff N, Wilson JN, Deane WM, Hoyt TW, Fowler BN, Gerety RL, Hoffman AR: Left main coronary artery embolism: A case report. Catheterization and Cardiovascular Diagnosis 1987 Nov-Dec;13(6):394-397.
- Maddoux GL, Ramo BW, Goss JE, Raff GL, **Heuser RR**, Shadoff N, Leatherman GF, Blake K, Wilson JN, Deane WM, Hoyt TW, Fowler BN, Gerety RL, Sansonetti E: Angina and vasospasm at rest in a patient with an anomalous left coronary system. Catheterization and Cardiovascular Diagnosis 1989 Feb;16(2):95-98.
- Goss JE, Ramo BW, Raff GL, Maddoux GL, **Heuser RR**, Shadoff N, Leatherman GF, Blake K: Power injection of contrast media during percutaneous transluminal coronary artery angioplasty. Catheterization and Cardiovascular Diagnosis 1989 Mar;16(3):195-198.
- Heuser RR**, Mehta SS: Holmium laser angioplasty after failed coronary balloon dilation: Use of a new solid-state, infrared laser system. Catheterization and Cardiovascular Diagnosis 1991 Jul;23(3):187-189.

- Heuser RR**, Mehta SS, Strumpf RK, Ponder R: Intracoronary stent implantation via the brachial approach: A technique to reduce vascular bleeding complications. *Catheterization and Cardiovascular Diagnosis* 1992 Apr;25(4):300-303.
- Strumpf RK, Mehta SS, Ponder R, **Heuser RR**: Palmaz-Schatz stent implantation in stenosed saphenous vein grafts: Clinical and angiographic follow-up. *American Heart Journal* 1992 May;123(5):1329-1336.
- Segal J, Kern MJ, Scott NA, King SB, Doucette JW, **Heuser RR**, Ofili E, Siegel R: Alterations of phasic coronary artery flow velocity in humans during percutaneous coronary angioplasty. *The Journal of the American College of Cardiology* 1992 Aug;20(2):276-286.
- Heuser RR**: The use of the Holmium: YAG laser in coronary disease: The utility of a unique lensed fiber catheter. *The Journal of Interventional Cardiology* 1992 Dec;5(4):293-300.
- Heuser RR**, Eagan JT, Strumpf RK: Angioscopy in coronary interventions. *Cardiology Intervention* 1992;2(4):23-28.
- Santiago O, Diethrich EB, **Heuser RR**, Gustafson G: What is the next step for the application of the Palmaz stent - the abdominal aorta? *Angiology* 1992;42:267-268.
- Heuser RR**, Mehta SS, Strumpf RK: The ACS RX™ flow support catheter as a temporary stent for dissection or occlusion during balloon angioplasty: Initial experience. *Catheterization and Cardiovascular Diagnosis* 1992 Sept;27(1):66-74.
- Diethrich EB, Santiago O, Gustafson G, **Heuser RR**: Preliminary observations on the use of the Palmaz stent in the distal portion of the abdominal aorta. *American Heart Journal* 1993 Feb;125(2):490-501.
- Heuser RR**: Lasers in coronary disease (commentary). *Catheterization and Cardiovascular Interventions* 1993;28:17.
- Eagan JT, Strumpf RK, **Heuser RR**: New treatment approach for chronic total occlusions of saphenous vein grafts: Thrombolysis and intravascular stents. *Catheterization and Cardiovascular Diagnosis* 1993 May;29(1):62-69.
- Heuser RR**, Strumpf RK, Diethrich EB, Eagan JT, Hardigan KR: Intraluminal diagnostics, the "guide" to the future. *Angiology* 1993;44:6.
- Heuser RR**, Strumpf RK, Eagan JT, Hardigan KR: Experience with four types of coronary stents. *Angiology* 1993;44:6-7.
- Heuser RR**: The use of a new wire in a 6-year-old coronary artery occlusion: The Jagwire™ recanalization guidewire. *Catheterization and Cardiovascular Diagnosis* 1993 Jun;29(2):173-176.
- Heuser RR**, Strumpf RK, Hardigan K: Use of the Doppler guidewire for intraluminal diagnosis to facilitate coronary intervention. *American Heart Journal* 1993 July; 126(1):213-218.
- Strumpf RK, **Heuser RR**, Eagan Jr JT: Angioscopy: A valuable tool in the deployment and evaluation of intracoronary stents. *American Heart Journal* 1993 Nov;126(5):1204-1210.
- Heuser RR**: Current status of coronary stents: Promises and disappointments. *Critical Issues* 1993;2:1-13.

- White CJ, Ramee SR, Collins TJ, Escobar AE, Karsan A, Shaw D, Jain SP, Bass TA, **Heuser RR**, Teirstein PS, Bonan R, Walter PD, Smalling RW: Coronary thrombi increase PTCA risk: Angioscopy as a clinical tool. *Circulation* 1996 Jan;93(2):253-258.
- Fischman DL, Leon MB, Baim D, Schatz RA, Penn I, Detre K, Savage MP, Veltri L, Ricci D, Nobuyoshi M, Cleman M, **Heuser RR**, Almond D, Teirstein P, Fish D, Colombo A, Brinker J, Moses J, Hirshfeld J, Bailey S, Ellis S, Rake R, Goldberg S: For the STRESS Trial Investigators: A randomized comparison of coronary stent placement and balloon angioplasty in the treatment of coronary artery disease. *The New England Journal of Medicine* 1994; 331:396-501.
- Aker UT, Kensey KR, **Heuser RR**, Sandza JG, Kussmaul III WG: Immediate arterial homostasis after cardiac catheterization: Initial experience with a new puncture closure device. *Catheterization and Cardiovascular Diagnosis* 1994 Mar;31(3):228-232.
- Flaherty JT, Pitt B, Gruber JW, Topol E, **Heuser RR**, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N, Brinker JA, Becker LC, Mancini CBJ, Weisfeldt ML, Werns SW: Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. *Circulation* 1994 May;89(5):1982-1991.
- Fenton SH, Fischman DL, Savage MP, Schatz RA, Leon MB, Baim DS, King SB, **Heuser RR**, Curry C, Rake RC, Goldberg S: Long-term angiographic and clinical outcome after implantation of balloon-expandable stents in aortocoronary saphenous vein grafts. *The Journal of the American College of Cardiology* 1994; Dec 15;74(12):1187-1191.
- Heuser RR**: Breaking the barrier: Stenting in acute myocardial infarction (commentary). *Catheterization and Cardiovascular and Diagnosis* 1994;33:46.
- Heuser RR**: A wire in every lesion (commentary). *Catheterization and Cardiovascular Interventions* 1994;33:197.
- Heuser RR**: All the glitters in not gold (commentary). *Catheterization and Cardiovascular Interventions* 1994 Dec;33(4):330.
- Savage MP, Fischman DL, Schatz RA, Teirstein PS, Leon MB, Baim DS, Ellis SG, Topol EJ, Hirshfeld JW, Cleman MW, Buchbinder M, Bailey S, **Heuser RR**, Walker CM, Curry RC Jr, Gebhardt S, Rake R, Goldberg S: For the Palmaz-Schatz Study Group: Long-term angiographic and clinical outcome after implantation of a balloon-expandable stent in native coronary circulation. *The Journal of the American College of Cardiology* 1994;24:1207-1212.
- Heuser RR**, Diethrich EB, Papazoglou C, Reynolds GT: Endoluminal grafting for percutaneous aneurysm exclusion in an aortocoronary saphenous vein graft: the first clinical experience. *Journal of Endovascular Surgery* 1995 Feb;2(1):81-88.
- Diethrich EB, **Heuser RR**, Tarlian H, Eckert J, Cardenas JR,: Endovascular techniques in adult aortic coarctation: the use of stents for native and recurrent coarctation repair. *Journal of Endovascular Surgery* 1995 May;(2)2:183-188.
- Aguirre FV, Topol EJ, Ferguson JJ, Anderson K, Blankenship JC, **Heuser RR**, Sigmon K, Taylor M, Gottlieb R, Hanovich G, Rosenberg M, Donohue TJ, Weisman H, Califf RM for the EPIC Investigators: Bleeding complications with the chimeric antibody to platelet glycoprotein IIb/IIIa integrin in patients undergoing percutaneous coronary intervention. *Circulation* 1995; June 15;91(12):2882-2890.

- Heuser RR:** Treatment alternatives for chronically occluded saphenous vein grafts. The Journal of Invasive Cardiology 1995 May;7(4):94-96.
- Kussmaul III WG, Buchbinder M, Whitlow PL, Aker UT, **Heuser RR**, King SB, Kent KM, Leon MB, Kolansky DM, Sandza JG: Rapid arterial homeostasis and decreased access site complications after cardiac catheterization and angioplasty: Results of a randomized trial of a novel hemostatic device. The Journal of the American College of Cardiology 1995 June;25(7):1685-1692.
- Wong SC, Baim DS, Schatz RA, Teirstein PS, King SB 3rd, Curry RC Jr, **Heuser RR**, Ellis SG, Cleman MW, Overlie P, et al. Immediate results and late outcomes after stent implantation in saphenous vs lesions: the multicenter US Palmaz-Schatz stent experience. The Palmaz-Schatz Study Group. Journal of the American College of Cardiology 1995 Sept;26(3):704-712.
- Cardenas JR, Strumpf RK, **Heuser RR:** Rotational artherectomy in restenotic lesions at the distal saphenous vein graft anastomosis. Catheterization and Cardiovascular Interventions 1995 Sept;36(1):53-57.
- Cohen DJ, Krumholz HM, Williams C, Baim DS, Brinker J, Cabin HS, **Heuser RR**, Leon MB, Moses J, Savage MP, Cleman M: In-hospital and one-year economic outcomes after coronary stenting or balloon angioplasty. Results from a randomized clinical trial. The Stent Restenosis Study Investigators. Circulation 1995 Nov.;1;92(9):2480-2487.
- Heuser RR:** A new breed of drug pusher? (commentary). Catheterization and Cardiovascular Interventions 1995;34:271.
- Heuser RR:** Recanalization of occluded SVGs: Is there light at the end of the graft? (commentary). Catheterization and Cardiovascular Diagnosis 1995 Dec;36(4):333-334.
- Heuser RR:** Percutaneous revascularization using endoluminal prostheses: Coming of age (commentary). Catheterization and Cardiovascular Diagnosis 1996 Feb;37(2):213-214.
- Kussmaul WG, Buchbinder M, Whitlow PL, Aker UT, **Heuser RR**, King SB, Kent KM, Leon MB, Kolansky DM, Sandza JG: Femoral artery hemostasis using an implantable device (AngioSeal™) after coronary angioplasty. Catheterization and Cardiovascular Diagnosis 1996 Apr;37(4):362-365.
- Heuser RR:** Stents in jackets: The latest in endovascular haute couture. Catheterization and Cardiovascular Diagnosis 1996 Jun;38(2):179.
- Heuser RR:** "Homemade" stents: Penny-wise or pound-foolish? (commentary) Catheterization and Cardiovascular Diagnosis 1996 Oct;39(2):197.
- Schultz RD, **Heuser RR**, Hatler C, Frey D: Use of c7E3 Fab in conjunction with primary coronary stenting for acute myocardial infarctions complicated by cardiogenic shock. Catheterization and Cardiovascular Diagnosis 1996 Oct;39(2):143-148.
- Heuser RR:** The Multi-Link stent: Another good idea. (commentary) Catheterization and Cardiovascular Diagnosis 1996 Dec;39(4):420.
- Kussmaul WG, Sandza JG, Kolansky DM, Leon MB, Kent KM, King SB, **Heuser RR**, Aker UT, Whitlow PL, Buchbinder M: Femoral artery hemostasis using an implantable device (Angio-Seal™) after coronary angioplasty. Catheterization and Cardiovascular Interventions 1996;37:362-365.

Heuser RR, Hartz RS: Embolization of IMA side branch for post-CABG ischemia. *Ann Thorac Surgery* 1997 Jun;63(6):1765-1766.

Heuser RR: Unlimiting our resources. (commentary) *Catheterization and Cardiovascular Diagnosis* 1997 Aug;41(4):415.

Krumholz HM, Cohen DJ, Williams C, Baim DS, Brinker J, Cabin HS, **Heuser RR**, Hirschfeld J, Leon MB, Moses J, Savage MP, Cleman M: Health after coronary stenting or balloon angioplasty: Results from the Stent Restenosis Study (STRESS). *The American Heart Journal* 1997 Sept;134(3):337-44.

Heuser RR: A Freudian stent? (commentary) *Catheterization and Cardiovascular Interventions* 1997;41:4.

Savage MP, Douglas JS, Fischman DL, Pepine CJ, King III SB, Werner JA, Bailey SR, Overlie PA, Fenton SH, Brinker JA, Leon MB, Goldberg S, **Heuser RR:** Stent placement compared with balloon angioplasty for obstructed coronary bypass grafts. *The New England Journal of Medicine* 1997;337:740-747.

Heuser RR, Lopez AN: Stenting, angioplasty and adjunctive treatments in acute myocardial infarction. *Cardiology Rounds* 1998;15:9:8-14.

Savage MP, Douglas Jr JS, Fischman DL, Pepine CJ, King SB, Werner JA, Bailey SR, Overlie PA, Fenton SH, Brinker JA, Leon MB, **Heuser RR**, Smalling R, Safian RD, Cleman M, Buchbinder M, Rake RC, Gebhardt S, Goldberg S, for the Stent Restenosis Study (STRESS) Investigators: Efficacy of coronary stenting versus balloon angioplasty in small coronary arteries. *The Journal of the American College of Cardiology* 1998;31:307-311.

Heuser RR: Outpatient coronary angiography: Indications, safety, and complication rates. *Herz* 1998 Feb;23(1):21-26.

Heuser RR: Garbage in, garbage out. (commentary) *Catheterization and Cardiovascular Interventions* 1998 Apr;43(4):402.

Heuser RR: Success with carotid stenting: A stroke of good luck or the wave of the future? (commentary) *Catheterization and Cardiovascular Interventions* 1998 May;44(1):7-8.

Heuser RR: Stentus Noninterruptus. (commentary) *Catheterization and Cardiovascular Interventions* 1998;43:253.

Woodfield SL, Lopez AN, **Heuser RR:** Fracture of Coronary Guidewire During Rotational Arterectomy with Coronary Perforation and Tamponade. *Catheterization and Cardiovascular Diagnosis* 1998 Jun;44(2):220-223.

Heuser RR, Lopez AN: Stenting, Angioplasty, and Adjunctive Treatments in Acute Myocardial Infarction. *Cardiology Review* 1998;15:8-14.

Heuser RR: Endovascular Haute Couture Revisited: Ready-to-Wear Comes of Age. (commentary) *Catheterization and Cardiovascular Interventions* 1998 Nov;45(3):314.

Heuser RR: The Cure for a Rainy Day. (commentary) *Catheterization and Cardiovascular Diagnosis* Dec 1998;45(4):421.

Heuser RR, Lopez A: Abdominal Aorta Aneurysm and ELG: A Review of a Treatment in Its Infancy. *Journal of Interventional Cardiology* 1998 December (II);6:591-602.

Heuser RR, Woodfield SL, Lopez AN: Obliteration of a Coronary Artery Aneurysm with a PTFE-Covered Stent: Endoluminal Graft for Coronary Disease Revisited. *Catheterization and Cardiovascular Interventions* 1999 Jan;46(1):113-116.

Heuser RR: The Right Place, the Right Time. *Catheterization and Cardiovascular Interventions* 1999 Apr;46(4):450-451.

Heuser RR: The growing role of stents in coronary artery disease. *Patient Care* May 1999: 43-58.

Teirstein PS, Mann III JT, Cundey PE, Schechter E, Jacobs WC, Grines CL, Stagaman DJ, Lansky AJ, Hultquist MA, Kusnick BA, **Heuser RR**, Kleinert HD, Popma J: Low versus high-dose recombinant Urokinase for the treatment of chronic saphenous vein graft occlusion. *The American Journal of Cardiology* 1999 June 15;83(12):1623-1628.

Heuser RR: Warming up to the reptilian heart (commentary). *Catheterization and Cardiovascular Interventions* 1999 Jul;47(3):360.

Heuser RR: Of Baling Wire and Bubble Gum. *Catheterization and Cardiovascular Interventions* 1999 August;47(4):495.

Heuser RR: Innovative Interventions. *Catheterization and Cardiovascular Interventions* 1999 Sept;48(1):113.

Cordero-Yordan H, Lopez AN, **Heuser RR:** Saphenous Vein Graft Intervention, *The Journal of Interventional Cardiology* 1999;12(6):495-497.

Cordero-Yordan H., Lopez AN, **Heuser RR:** Carotid Artery Percutaneous Transluminal Angioplasty and Stenting; *The Journal of Interventional Cardiology* 1999;12:499-504.

Heuser RR, Cordero-Yordan H: Carotid Artery Percutaneous Transluminal Angioplasty and Stenting: Indications, Technical Approach, and Complications. *The Journal of Interventional Cardiology*. 1999;12(6): 499-504.

Heuser RR: At Arms' Length: Should We Teach Radial and Brachial Access Techniques? *Catheterization and Cardiovascular Interventions* 2000 Jan;49(1):38.

Heuser RR: Coronary Lesions: The Good, the Bad, and the Ugly, *Catheterization and Cardiovascular Interventions* 2000 Jan;49(1):112.

Oesterle SN, Sanborn TA, Ali N, Resar J, Ramee SR, **Heuser RR**, Dean L, Knopf W, Schofield P, Schaer GL, Reeder G, Masden R, Yeung AC, Burkhoff D: Percutaneous transmyocardial laser revascularization for severe angina: the PACIFIC randomized trial. *The Lancet* (356) November 28, 2000;1705-1710.

Heuser RR, Houser F., Culler SD., Becker ER, Battaglia SL, Tarkington L, Simon AW: A Retrospective Study of 6,671 Patients comparing Coronary Stenting and Balloon Angioplasty; *The Journal of Invasive Cardiology* 2000;July 2000.

Heuser RR: Delivery Systems for Endoluminal Grafting of Abdominal Aortic Aneurysms: A Square Peg for a Round Hole? (commentary). *Catheterization and Cardiovascular Interventions* 2000 Jul;50(3):361.

Heuser RR, Cordero H, Gupta N, Underwood PL, Gogte ST: Intracoronary Autologous Blood to Seal a Coronary Perforation; *Herz* 2001; March 26;(2):157-160.

Heuser RR, Lopez A, Kuntz R, Reduto L, Badger R, Coleman P, Whitlow P, Iannone LA, Safian R, Yeung A, Moses J: SMART: The MicroStent's Ability to Limit Restenosis Trial. Catheterization and Cardiovascular Interventions 52:269-277 2001

Cordero H, Warburton KD, Underwood PL, **Heuser RR**: Initial Experience and Safety in the Treatment of Chronic Total Occlusions with Fiberoptic Guidance Technology: Optical Coherent Reflectometry. Catheterization and Cardiovascular Interventions Oct 2001; 54(2):180-187.

Heuser RR: If It Can Keep Eggs Off the Frying Pan... (commentary) Catheterization and Cardiovascular Interventions May, 2001; 53:5.

Heuser RR, Cordero H, Schroeder W, Weirick BA, Hatler CW, Underwood PL: Fiberoptic Guidance System for Treatment of Chronic Total Occlusions: Single Center Experience. Journal of the American College of Cardiology; February 2001 (37) Supplement A: 1158-38.

Heuser RR, Morales PA: Across the Great Divide (commentary) Catheterization and Cardiovascular Interventions. October 2001; 54(2):264.

Ellis SG, Mooney M, Talley DJ, Silber S, Teirstein PS, Rodriguez AR, Sanborn TA, Feldman T, Leon M, Collins TJ, Wilentz JR, Saucedo JF, Leimgruber PP, Pichard A, Cohen DJ, Ramee SR, **Heuser RR**, Smalling RW, Kuntz RE, Gershony G: Assessment of the Safety and Efficacy of the Duett™ Vascular Hemostasis Device - Final Results of the Seal Trial. American Heart Journal April 2002; 143(4):612-9.

Morales PA, **Heuser RR**: Chronic Total Occlusions: Experience with Fiber-Optic Guidance Technology - Optical Coherence Reflectometry. Journal of Interventional Cardiology; December 2001; 14:6:611-616.

Morales PA, **Heuser RR**: Peripheral Vascular Disease: Perspectives on Aortoiliac, Renal, and Femoral Treatments Using Catheter-Based Techniques. Journal of Interventional Cardiology; December 2001; 14:6:629-637.

Heuser RR, Laskey W, Lansky A, Popma J, Bonan R: Beta Radiation in Lesions >15 mm: A START Subgroup. Journal of the American College of Cardiology. (In Press)

Philip A. Morales, M.D., **RR Heuser**, Embolic Protection Devices. Journal of Interventional Cardiology; December 2002; 15:485-490.

Philip A. Morales, M.D., and **RR Heuser**, Guiding Catheter Aspiration to Prevent Embolic Events During Saphenous Vein Graft Intervention. Journal of Interventional Cardiology; December 2002; 15:491-498.

Jeffrey J. Popma, Mohan Suntharalingam, Alexandra J. Lansky, **Richard R. Heuser**, Burton Speiser, Paul S. Teirstein, Vincent Massullo, Theodore Bass, Randall Henderson, Sigmund Silber, Peter von Rottkay, Raoul Bonan, Kalon K.L. Ho, Allison Osattin, MPH; Richard E. Kuntz, for the Stents And Radiation Therapy (START) Investigators, Randomized Trial of 90 SR/90 Y B-Radiation Versus Placebo Control for Treatment of In-Stent Restenosis. Circulation; August 2002; 106:1090-1096.

EDITED TEXTBOOKS:

Heuser RR: *Peripheral Vascular Stenting for the Cardiologist*, Martin Dunitz, Ltd Publishing, 1999

Heuser RR, Henry M: *Peripheral Vascular Interventions*, Martin Dunitz, Ltd Publishing; March 2002

TEXTBOOK CHAPTERS:

- Heuser RR:** Coronary angioplasty for acute mitral regurgitation due to myocardial infarction. In: *Ischemic Mitral Incompetence*. Vetter HO, Hetzer R, Schumtizer H, eds. New York, Springer Verlag, 1991:99-114.
- Murphy-Chutorian D, Knopf W, Moses J, **Heuser RR:** Selected clinical cases using the eclipse laser angioplasty system. In: *Primer of Laser Angioplasty*, 2nd ed., Ginsburg R, Geschwind H, eds. Futura Publishing Co., 1991:309-324.
- Heuser RR:** Experience with different coronary stents. In: *Endoluminal Stenting*. Sigwart U, ed. London, W.B. Saunders Company Ltd., 1996:338-345.
- Heuser RR:** Peripheral stenting for the cardiologist. In: *Frontiers in Interventional Cardiology*. Beyar, Keren, Leon M, Serruys P, eds. London, Martin Dunitz Limited, 1997:377-382.
- Erbel R, O'Neil W, **Heuser RR:** Long Lesion. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:48-53.
- Heuser RR, Schatz R, Kimura T:** Tortuous RCA. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:76-80.
- Heuser RR, Schatz R, Kimura T:** Tortuous Circumflex. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:81-87.
- Heuser RR, Kern M:** Residual stenosis: Vein Graft. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:549-551.
- Nobuyoshi M, **Heuser RR, Morice MC:** New filling defects. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:555-557.
- Heuser RR, Schatz R, Nobuyoshi M:** Stent: Filling Defects. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:599-600.
- Heuser RR, Holmes D, Roubin G:** Free Perforation. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:615-617.
- Heuser RR, Holmes D, Roubin G:** Contained Perforation. In: *Tough Calls in Interventional Cardiology*. Safian RD, Freed M, eds. Birmingham, MI, Physician's Press, 1997:618-620.
- Heuser RR, Lass, T, Prebil B, Reid DB:** Computerized three-dimensional intravascular reconstruction. In: *Coronary Stenosis Morphology: Analysis and Implication*. Klein LW, ed. Chicago, IL, Kluwer Academic Publishers, 1997:103-120.
- Heuser RR, Lopez AN:** Introduction. In: *Peripheral Vascular Stenting for Cardiologists* Martin Dunitz Ltd. 1999:1-4.
- Woodfield SL, **Heuser RR:** Angiographic anatomy of the peripheral vasculature and the noninvasive assessment of peripheral vascular disease. In: *Peripheral Vascular Stenting for Cardiologists* Martin Dunitz Ltd. 1999:5-16.
- Heuser, RR, Gupta N, Lopez AN:** AVE Stents. In: *Strategic Approaches in Coronary Interventions, Second Edition*, Lippincott Williams & Wilkins Publishers, 2000.

Heuser RR, Cordero-Yordan H: Carotid Artery Interventions. In: *Practical Handbook of Advanced Interventional Cardiology*, Nguyen T, Saito S, Hu D, Feldman T, Armonk, New York, Futura Publishing Company; In press.

Heuser RR: *PTFE-Covered Stents*. In: Handbook of Local Drug Delivery, Martin Dunitz Ltd. Publishing; in press.

Heuser RR, Underwood PL: *Covered Stents in Peripheral Vascular Diseases*. In: Textbook of Vascular Stenting Interventions: in press.

Heuser RR: *Certification and Training in Peripheral Vascular Intervention*. In: Textbook of Vascular Stenting Interventions: in press.

Cordero H, **Heuser RR**: *The cardiologist and peripheral intervention*. In: Practical Interventional Cardiology 2nd Edition, Martin Dunitz Ltd, Publishing: 2002:349-359.

White CW, **Heuser RR**: *The Covered Stent-Graft: Treatment of an Arteriovenous Fistula*. In: Fifty Cases in Peripheral Intervention. Martin Dunitz Ltd. Publishing: 2002:71-73.

ABSTRACTS:

Fuchs RM, **Heuser RR**, Brinker JA: Limitations of pulmonary capillary wedge V waves in diagnosing mitral regurgitation. Presented at XI InterAmerican Congress of Cardiology; September 1980.

Heuser RR, Brinker JA: Acute effects of protamine of cardiac function. Circulation 1980; 62(3): I-II.

Heuser RR, Brinker JA: Cardiac catheterization in aortic stenosis: "A Necessary Evil". Clinical Research 1981; 29:206A.

Alexander E, **Heuser RR**, Weiss JL, Firestein GS, Leitl G, Wagner Jr HN, Stevens MB, Becker LC: Sclerodermal heart disease: Evidence for cold induced abnormalities of myocardial perfusion and function. Circulation 1981; 64(4): IV-24.

Heuser RR, Becker LC, Flaherty J: Extensive myocardial infarction associated with minor electrocardiographic abnormalities: Value of admission scintigraphy. Circulation 1981; 64(4):IV-85.

Heuser RR, Goss JE, Ramo BW, Raff GL, Maddoux GL: Coronary angioplasty in Albuquerque: Report of the first large series and preliminary follow-up. Presented at the American College of Physicians, Albuquerque, New Mexico; December 1984.

Heuser RR, Goss JE, Ramo BW, Raff GL, Maddoux GL: Coronary angioplasty in patients with acute mitral regurgitation due to myocardial infarction: A nonsurgical treatment to preserve mitral valve integrity. Presented at the American Heart Association Meeting, Washington, DC; November 1985.

Heuser RR, Maddoux GL, Goss JE, Ramo BW, Raff GL: Treatment of cardiogenic shock with percutaneous coronary angioplasty: The therapy of choice. Presented at the American College of Cardiology Meeting, Atlanta, Georgia; March 1986.

Heuser RR, Maddoux GL, Goss JE, Ramo BW, Raff GL, Shadoff N: Coronary angioplasty in the elderly: A low risk option. Presented at the World Congress of Cardiology, Washington, DC; September 1986.

Heuser RR, Goss JE, Ramo BW, Raff GL, Maddoux GL, Shadoff N: Repeat coronary angioplasty: A highly successful and low risk procedure. Presented at the World Congress of Cardiology, Washington, DC; September 1986.

Raff GL, **Heuser RR**, Maddoux GL, Goss JE, Ramo BW, Archuleta B: Thallium treadmill testing accurately predicts restenosis following percutaneous transluminal coronary angioplasty. Presented at the World Congress of Cardiology, Washington, DC; September 1986.

Leatherman GF, Goss JE, Ramo BW, Raff GL, Maddoux GI, **Heuser RR**, Shadoff N, Blake K: Bucindolol improves exercise tolerance and cardiac function in patients with congestive heart failure. Presented at American College of Physicians Meeting, Albuquerque, New Mexico; December 1989.

Heuser RR, Goss JE, Ramo BW, Raff GL, Maddoux GI, Shadoff N, Leatherman GF, Blake K: A Randomized, double-blind trial of recombinant human superoxide dismutase (h-SOD) in patients undergoing PTCA for acute MI. Presented at the American College of Physicians Meeting, Albuquerque, New Mexico; December 1989.

Heuser RR, Goss JE, Ramo BW, Raff GL, Maddoux GI, Shadoff N, Leatherman GF, Blake K: Laser-assisted coronary angioplasty: Report of initial experience. Presented at the American College of Physicians Meeting, Albuquerque, New Mexico; December 1989.

Blake K, Goss JE, Ramo BW, Raff GL, Maddoux GL, **Heuser RR**, Shadoff N, Leatherman GF: Adenosine for acute treatment of SVT. Presented at the American College of Physicians Meeting, Albuquerque, New Mexico; December 1989.

Werns S, Brinker JA, Gruber J, Rothbaum D, **Heuser RR**, George B, Burwell L, Kereiakes D, Mancini GB, Flaherty J: A Randomized double-blind trial of recombinant human superoxide dismutase (h-SOD) in patients undergoing PTCA for acute MI. *Circulation* 1990 (IV):11-113.

Heuser RR, Schatz RA, Diethrich EB, Ponder R, Waters DB, Skeete R: Placement of the Palmaz balloon-expandable intraluminal stent in iliac arteries: A single center experience. *Radiology* 1991;181 (supple):295.

Heuser RR, Diethrich EB, Waters DB: Abdominal aortic stenosis: Application of Endovascular stents. *Radiology* 1991;181(supple):295.

Leon MB, Ellis SG, Pichard AD, Baim DS, **Heuser RR**, Schatz RA: Stents may be the preferred treatment for focal aortocoronary vein graft disease. *Circulation* 1991;84 (supple II): II-249.

Heuser RR, Dorsey R, Mehta S: The "Smart Needle" - a new device for vascular access. Presented at Congres Internationale d'Angiologie, Paris, France; April 1991.

Heuser RR, Mehta S, Ponder R: Stent implantation for stenosis in saphenous vein bypass grafts. Presented at Congres Internationale d'Angiologie, Paris, France; April 1991.

Ravi R, **Heuser RR**, Diethrich EB, Mehta S, Ponder R: Application of endovascular stents in the abdominal aorta. Presented at Congres Internationale d'Angiologie, Paris, France; April 1991.

Mehta S, **Heuser RR**, Siegel RM, Willoughby S: Initial report of a new, pulsed, solid-state laser system for percutaneous coronary angioplasty. Presented at Congres Internationale d'Angiologie, Paris, France; April 1991.

Mehta S, **Heuser RR**, Ponder R: Use of the Palmaz-Schatz balloon-expandable intracoronary stent for the treatment of coronary artery disease. Presented at the American College

of Chest Physicians, Arizona Chapter, Tucson, Arizona; September 1991.

Morton JK, Segal J, Ofili E, **Heuser RR**, Scott N, Aguirre F, St. Vrain J, Labovitz A, FLOMAP Study Group, SLU: A new method for quantitating coronary collateral flow in patients during coronary angioplasty using the Doppler guidewire. (poster) Presented at the American Heart Association Meeting, Anaheim, California; November 1991.

Heuser RR: Use of Holmium: YAG laser in coronary disease. Presented at the International Society for Optical Engineering - Biomedical Optics 1991. Los Angeles, California; January 1992.

Heuser RR, Strumpf RK, Walker CM, Cleman MW, Schatz RA: Coronary stenting in unstable angina: No greater risk than in stable angina. The Journal of the American College of Cardiology 1992;19:178A.

Whitlow PL, Gaspard P, Kent KM, Baim DS, Chapman JB, **Heuser RR**, Knopf WK: Improvement of coronary dissection with a removable flow support catheter: Acute results. The Journal of the American College of Cardiology 1992; 19:217A.

Teirstein PS, Schatz RA, Rocha-Singh KJ, Wong SC, Strumpf RK, **Heuser RR**: Coronary stenting with angioscopic guidance. The Journal of the American College of Cardiology 1992; 19:223A.

Donohue TJ, Kern MJ, Aguirre FV, Bell C, Penick D, Segal J, Ofili E, **Heuser RR**: Determination of the hemodynamic significance of angiographically intermediate coronary stenosis by intracoronary Doppler flow velocity. The Journal of the American College of Cardiology 1992; 19:242A.

Leon MB, Kent KM, Baim DS, Walker CM, Cleman MW, Buchbinder M, **Heuser RR**, Curry C, Schatz RA, JJIS Stent Investigators: Comparison of stent implantation in native coronaries and saphenous vein grafts. The Journal of the American College of Cardiology 1992; 19:263A.

Heuser RR, Diethrich EB, Strumpf RK, Ponder R: Chronic iliac occlusions: Palmaz stents are the treatment of choice. Circulation 1992; 86(suppl):I-634.

Heuser RR, Strumpf RK: Coronary stenting of ostial coronary artery stenosis: Intravascular ultrasound guidance. Presented at Cardiostim 1992, Nice, France; June 1992.

Heuser RR, Strumpf RK: The use of angiography with coronary stenting. Presented at Cardiostim 1992, Nice, France; June 1992.

Heuser RR, Diethrich EB, Strumpf RK, Ponder R: Chronic iliac occlusions: Intraluminal stents are the treatment of choice. Presented at XIV Congress of European Society of Cardiology, Barcelona, Spain; August 1992.

Heuser RR, Strumpf RK: Angioscopy with coronary stenting: Is it helpful? Presented at XIV Congress of European Society of Cardiology, Barcelona, Spain; August 1992.

Heuser RR: Intravascular coronary artery stents. Presented at the American College of Surgeons, postgraduate courses, New Orleans, Louisiana; October 1992.

Heuser RR, Brinker JA, Weiner BH for Strecker Stent Investigators: Strecker coronary stent: Initial multicenter experience. The Journal of the American College of Cardiology 1993; 21:293A.

- Fischman D, Savage M, Leon M, Schatz RA, **Heuser RR**, Goldberg S: Acute and long-term results of coronary stenting for complex intimal dissections following balloon angioplasty. *The Journal of the American College of Cardiology* 1993; 21:178A.
- Schatz RA, Penn IM, Baim DS, Nobuyoshi M, Colombo A, Ricci DR, Cleman MW, Goldberg S, **Heuser RR**, Almond D, Fish D, Moses J, Gallup D, Detre K, Leon MB for the STRESS Investigators: Stent Restenosis Study (STRESS) analysis of in-hospital results. *Circulation* 1993; 88:I-594.
- Heuser RR**, Hardigan K, Strumpf R, Eagan J, Aultman R, Prebil B, Waters K: Single Center Palmaz "Biliary" Stent Experience in Coronary Arteries and Saphenous Vein Grafts. *Circulation* 1993; 88:(4) Part II.
- White CJ, Ramee SR, Collins TJ, Jain SP, Escobar A, Soni D, Bass TA, **Heuser RR**, Teirstein PS, Bonan R, Nesto RW, Walter PD, Smalling RW, Knopf WD: Angioscopically detected coronary thrombus correlates with adverse PTCA outcome. *Circulation* 1993; 88:I-596.
- Kussmaul WG, Buchbinder M, Whitlow PL, Aker UT, **Heuser RR**, King SB, Sandza JG, Kensey KR: Randomized trial of a new hemostatic puncture closure device. *Circulation* 1993; 88:I-72.
- Heuser RR**, Hardigan KR, Strumpf RK, Eagan JT: Single-center Palmaz biliary stent experience in coronary arteries and saphenous veins. *Circulation* 1993; 88:I-308.
- Fortuna R, **Heuser RR**, Garratt KN, Schwartz R, Buchbinder M: Wiktor intracoronary stent: Experience in the first 101 vein graft patients. *Circulation* 1993; 88:I-505.
- Teirstein PS, Schatz RA, Rocha-Singh KJ, DeNardo SJ, Morris N, Strumpf RK, **Heuser RR**: Angioscopy of coronary stents at follow-up. *The Journal of the American College of Cardiology* 1993; 21:134A.
- Thompson MA, Brinker JA, **Heuser RR**, Weiner BH, Hess K, Ross AM, Segal J, The George Washington University Quantitative Analysis Core Laboratory for the Strecker Stent Investigators: Balloon-expandable stent size versus inflation pressure: The Strecker experience. *The Journal of the American College of Cardiology* 1993; 21:293A.
- Heuser RR**, Hardigan K, Strumpf R, Eagan J, Aultman R, Prebil B, Waters K: Single Center Palmaz "Biliary" Stent Experience in Coronary Arteries and Saphenous Vein Grafts. Presented at the American Heart Association Scientific Session; October 1993.
- Heuser RR**, Strumpf RK, Ramirez-Cardenas J, Schmidt J: Experience with seven stent models in the coronary arteries and vein grafts. *Journal Interventional Cardiology* 1994; 7:90.
- Fischman D, Savage M, Leon MB, Schatz R, Baim D, Penn I, Detre K, **Heuser RR**, Ricci D, Fish D, Rake R, Gebhardt S, Goldberg S for the STRESS Investigators, Jefferson Medical College, Philadelphia, PA: Acute and late angiographic results of the Stent Restenosis Study (STRESS). *The Journal of the American College of Cardiology* 1994 (special issue): 60A.
- Segal J, Reiner JS, Thompson MA, Brinker JA, **Heuser RR**, Weiner BH, Ross AM, The George Washington University Quantitative Analysis Core Laboratory for the Strecker Stent Investigators: Residual stenosis following coronary stenting do not predict restenosis in the Strecker stent. *The Journal of the American College of Cardiology* 1994 (special issue):134A.
- Carrozza JP, Kuntz RE, Leon MB, Schatz RA, Brinker J, Chuang YC, **Heuser RR**, Baim DS: Demographic, angiographic and procedural factors associated with late angiographic outcome in the STRESS Trial: A multivariable analysis. *Circulation* 1994; 90:I-18.

Garratt KN, White CJ, Buchbinder M, Whitlow PK, **Heuser RR** on behalf of the American Wiktor Stent Investigators: Wiktor stent placement for unsuccessful coronary angioplasty. *Circulation* 1994; 90:1-279.

Heuser RR, Cleman M, Cabin H, Wong SC, Chuang YC, Bailey S, Nobuyoshi M, Almond D, Schatz R: The LAD subgroup in the Stent Restenosis Study: Early and late angiographic and clinical outcomes. *Circulation* 1994; 90:1-323.

Savage M, Fischman D, Rake R, Hirshfeld J, Penn I, **Heuser RR**, Shaknovich A, Moses J, Ricci D, Goldberg S: Effect of lesion morphology on angiographic outcome after balloon angioplasty and coronary stenting: Results from the STRESS Trial. *Circulation* 1994; 90:1-324.

Kussmaul WG, Sandza JG, **Heuser RR** for the Anglo-Seal™ in patients at high risk of bleeding complications. *Catheterization and Cardiovascular Interventions* 1994; 32:98.

Sketch Jr. MH, Wong SC, Chuang YC, Phillips HR, **Heuser RR**, Savage M, Stack RS, Baim DS, Schatz RA, Leon MB for the JJIS Stent Investigators: Progressive deterioration in late (2-Year) clinical outcomes after stent implantation in saphenous vein grafts: The multicenter JJIS experience. *The Journal of the American College of Cardiology* 1995 (special issue): 79A.

Savage M, Douglas J, Fischman D, Fenton S, King S, Pepine C, Bailey S, Overlie P, Werner J, Leon M, **Heuser RR**, Brinker J, Buchbinder M, Smalling R, Snead D, Rake R, Gebhardt S, Kerensky R, Wargovich T, Goldberg S, SAVED Trial Investigators: Coronary stents versus balloon angioplasty for aortocoronary saphenous vein bypass graft disease: Interim results of a randomized trial. *The Journal of the American College of Cardiology* 1995 (special issue):79A.

Wong SC, Chuang YC, Detre K, Pichard AD, Schatz RA, **Heuser RR**, Kent KM, Leon MB: Is the anti-restenosis effect of stent (vs PTCA) greater in LAD vessels? A subgroup analysis of the Stent Restenosis Study. *The Journal of the American College of Cardiology* 1995 (special issue):374A.

Primack DS, **Heuser RR**, Kuntz RE, Waters K, Swiderski K: Coronary stenting performed via brachial access is more economical. Presented at the American College of Cardiology 44th Annual Scientific Session, New Orleans, Louisiana; March 1995.

Primack DS, **Heuser RR**, Kuntz RE, Waters K, Swiderski K: Coronary stenting performed via brachial access is more economical. Presented at the 18th Annual Meeting of the Society for Cardiac Angiography and Interventions, Orlando, Florida; May 1995.

Heuser RR, Reynolds GT, Papazoglou C, Diethrich EB: Endoluminal grafting for percutaneous aneurysm in an aortocoronary saphenous vein graft: The first human experience. Presented at the 18th Annual Meeting of the Society for Cardiac Angiography and Interventions, Orlando, Florida; May 1995.

Garratt KN, Schwartz RS, Holmes Jr DR, White CJ, Buchbinder M, Whitlow PL, **Heuser RR** on behalf of the American Wiktor Stent Investigators: Placement of Wiktor coronary stents for management of unsuccessful coronary angioplasty: The American Experience. Presented at the 1st International Meeting on Interventional Cardiology, Jerusalem, Israel; June 1995. *The Journal of Invasive Cardiology* 1995; 7 (supple)35C.

Heuser RR, Reynolds GT, Papazoglou C, Diethrich EB, Mukherjee R: Compassionate endoluminal grafting for percutaneous aneurysm exclusion in aortocoronary saphenous vein

grafts. Presented at the XVIIth Congress of the European Society of Cardiology, Amsterdam, The Netherlands; August 1995. *Journal of Endovascular Surgery* 1995; 2:81-88.

Heuser RR, Wong SC, Chuang YC, Detre K, Pichard AD, Schatz RA, Kent KM, Leon MB for the STRESS investigators: The LAD subgroup in the Stent REStenosis Study (STRESS): The most pronounced anti-restenosis effect of stenting. Presented at the XVIIth Congress of the European Society of Cardiology, Amsterdam, The Netherlands; August 1995.

Heuser RR, Reynolds GT, Papazoglou C, Diethrich EB, Mukherjee R: Compassionate endoluminal grafting for percutaneous treatment of aortocoronary SVG disease. *The Journal of the American College of Cardiology* 1996; 27:179A.

Savage M, Fischman D, Rake R, Schatz R, Penn I, Nobuyoshi M, Moses J, **Heuser RR**, Gebhardt S, Goldberg S for the STRESS Trial Investigators: Elective coronary stenting versus balloon angioplasty in smaller native coronary arteries: Results from STRESS. *The Journal of the American College of Cardiology* 1996; 27:253A.

Leon MB, Ellis SG, Moses J, King SB, **Heuser R**, Kent KM, Goldbert S, Fischman D, Senerchia C, Lanoue AS, Kuntz RE: Interim Report from the Reduced Anticoagulation Vein Graft Stent (RAVES) Study. Presented at the American Heart Association 69th Scientific Session, New Orleans, Louisiana; November 1996.

Savage M, Fischman D, Slota P, Rake R, Leon M, Schatz R, Moses J, Penn I, Nobuyoshi M, **Heuser RR**, Goldberg S: Coronary intervention in the diabetic patient: Improved outcome following stent implantation versus balloon angioplasty. Presented at the American College of Cardiology 46th Annual Scientific Session, Anaheim, California; March 1997.

Dean LS, **Heuser RR**, Moore PB, Rogers EW, Roubin GS, Leon MB, Voorhees WD, Holmes Jr DR, O'Shoughnessy CD, Zidar JP: Stenting in small vessels: A re-evaluation using the GR-II intracoronary stent, a Cook Registry Study. Presented at the American College of Cardiology 46th Annual Scientific Session, Anaheim, California; March 1997.

Heuser RR: Live Demonstrations. Presented at the 1997 Japanese Society of Interventional Cardiology Live Demonstration Symposium, Kokura, Japan; May 1997.

Heuser RR: Influence of minimally invasive coronary artery bypass surgery. Presented at the XIXth Congress of the European Society of Cardiology, Stockholm, Sweden; August 1997.

Heuser RR, Kuntz RE, Lansky AJ, Whitlow PL, Sanfian RD, Yeung AC, Senerchia C, Cutlip DE, Pino-Mauch B, Pedan A: The SMART Trial: Acute outcome indicates superior efficacy with the AVE Stent. Presented at the XIXth Congress of the European Society of Cardiology, Stockholm, Sweden; August 1997.

Heuser RR, Kuntz R, Popma J, Pino-Mauch B, Miller R, Yeung A, Badger R: The SMART Trial: 30-day outcome indicates superior efficacy with the AVE Stent. Presented at the Transcatheter Cardiovascular Therapeutics Symposium, Washington, DC; September 1997.

Heuser RR: Influence of modern bypass surgery on PTCA. Presented at 20 Years of PTCA - Back to the Cradle, Zurich, Switzerland; September 1997.

Heuser RR: An overview of new stent designs: Lesion specific stenting. Presented at Coronary Interventions 1997, LaJolla, California; October 1997.

Heuser RR: Covered stents: Clinical needs, future prospects. Presented at Coronary Interventions 1997, LaJolla, California; October 1997.

- Heuser RR**, Kuntz RE, Lansky AJ, Whitlow PL, Safian RD, Yeung AC, Senerchia C, Cutlip DE, Pino-Mauch B, Pedan A: The SMART Trial: Acute outcome indicates superior efficacy with the AVE Stent. American Heart Association 70th Scientific Sessions, Orlando, Florida; November 1997.
- Heuser RR**: Is carotid artery PTA superior to surgery? Presented at the International Course on Interventional Cardiology, Frankfurt, Germany; December 1997.
- Heuser RR**, Kuntz R, Popma J, Pino-Mauch B, Miller R, Yeung A, Badger R: The SMART Trial: 30-day outcome indicates superior efficacy with the AVE Stent. Presented at the American College of Cardiology 47th Annual Scientific Session, Atlanta, Georgia; April 1998.
- Heuser RR**, Kuntz R, Lansky, A, Kalon KLH, Reduto L, Badger R, Iannone LA, Whitlow P, Vlietstra R: A comparison of the Long AVE Micro™ Stent II and the Palmaz-Schatz® Stent: A SMART trial registry. Presented at the American College of Cardiology 47th Annual Scientific Session, Atlanta, Georgia; April 1998.
- Heuser RR**, Houser F, Battaglia S, Tarkington L, Simon A, Culler S, Becker E: Repeat procedure rates in percutaneous interventions: Is there added benefit of atherectomy or stenting? Presented at Transcatheter Cardiovascular Therapeutics, Washington, DC; October 1998.
- Heuser RR**, Lopez AN, Stoerger H, Reifart N: Coronary artery application of an endoluminal polytetrafluorethylene stent graft: Two center experience with the JOMED® JoStent®. Presented at American Heart Association, Dallas, Texas; November 1998.
- Ramee SR, Baim DS, Popma JJ, Ho KKL, Cutlip DE, Lanoue AS, Dandreo KJ, Schatz RA, Sharma SK, Kuntz RE, **Heuser RR**: A randomized, prospective, multi-center study comparing intracoronary Urokinase to Rheolytic thrombectomy with the POSSIS AngioJet Catheter for intracoronary thrombus: Final results of the VeGAS 2 Trial. Presented at American Heart Association, Dallas, Texas; November 1998.
- Heuser RR**, Lopez AN: Coronary Artery Disease Revascularization with the JOMED JoStent®: A Two Center Experience. Presented at American College of Cardiology, New Orleans, Louisiana; March 1999.
- Lansky AJ, Popma JJ, Mehran R, Hanzel G, Cutlip D, Ho KK, Proctor B, Hapji JA, Kuntz RE, Baim DS, **Heuser RR**, Midel M, O'Shaughnessy C, Leon MB: Tubular Slotted Stents: A "Breakthrough Therapy" for Women Undergoing Coronary Interventions. Pooled Results from the STARS, ACSSENT, SMART, and NIRVANA Randomized Clinical Trials. Presented at American College of Cardiology, New Orleans, Louisiana; March 1999.
- Oesterle SN, Yeung A, Ali N, Dean LS, **Heuser RR**, Knopf WD, Masden R, Ramee SR, Reeder GS, Resar J, Sanborn TA, Schaer GL, Schofield PM: The CardioGenesis Percutaneous Myocardial Revascularization (PMR) Randomized Trial: Initial Clinical Results. Presented at American College of Cardiology, New Orleans, Louisiana; March 1999.
- Heuser RR**: New Findings of PMR and Gene Therapy. Presented at 4th Asian-Pacific Society of Interventional Cardiology Conference and 8th Live Demonstration in Kokura, Kokura, Japan; May 1999.
- Heuser RR**: The Use of Covered Coronary Stents: A new Therapy in Evolution. Presented at 4th Asian-Pacific Society of Interventional Cardiology Conference and 8th Live Demonstration in Kokura, Kokura, Japan; May 1999.
- Heuser RR**: Radiofrequency Enhancement of Intramyocardial Gene Therapy. Presented at the Second Annual Symposium Angiogenesis and DMR Agenda, Washington, DC; June 1999.

Heuser RR: Covered Stents for Coronary Artery Disease: Early Results of Clinical Trials. Presented at the Sixth Annual Mayo Interventional Cardiology Symposium, Napa Valley, California; July 1999.

Heuser RR: Peripheral Vascular Disease Management: New Devices, New Drugs, New Approaches. Presented at the Sixth Annual Mayo Intervention Cardiology Symposium, Napa Valley, California; July 1999.

Heuser RR: Radiofrequency PMR: A Unique System for Simultaneous Radiofrequency PMR and Gene Therapy. Presented at the XXist Congress of the European Society of Cardiology, Barcelona, Spain; August 1999;20;2828.

Heuser RR: Do Coronary Stents Improve Clinical Outcomes? A retrospective study comparing stent device utilization and adverse outcomes. Presented at the XXist Congress of the European Society of Cardiology, Barcelona, Spain; August 1999.

Oesterle SN, Sanborn TA, Ali N, Resar J, Ramee SR, **Heuser RR**, Dean L, Knopf W, Schofield P, Schaer GL, Reeder G, Masden R, Yeung AC, Burkhoff D: Percutaneous transmyocardial laser revascularization for severe angina: the PACIFIC randomized trial. Presented at the 72nd Scientific Session American Heart Association, Atlanta, Georgia, November 1999.

Sameer Mehta, **Heuser RR**, Salvatore BL, Simon AW, Culler SD, Becker ER: Evolving Patterns of Coronary Stenting: Report from a Large National Database. Presented at the 49th Annual Scientific Session American College of Cardiology, March 2000.

Heuser RR: Future of Covered Stents and Endoluminal Grafts. Presented at the GET International Endovascular Peripheral Course in Monaco; May 2000.

Heuser RR, Heilbrunn S, Fogarty TJ, Motarjeme A: Establishing an Integrated Vascular Center. Presented at the Eighth New Devices Seminar for the Cardiac and Peripheral Vascular Laboratories in Orlando, Florida; July 2000.

Heuser RR, Covered Stents: New Options & Strategies. Presented at the Eighth New Devices Seminar for the Cardiac and Peripheral Vascular Laboratories in Orlando, Florida; July 2000.

Heuser RR, Waksman R, White CJ: Advanced Coronary Workshop: The experts discuss techniques and equipment on the Progress Occurring with Stent Grafts & Brachytherapy for the Coronary and Bypass Graft Circulation. Presented at the Eight New Devices Seminar for the Cardiac and Peripheral Vascular Laboratories in Orlando, Florida; July 2000.

Heuser RR, Cordero H, Underwood PL, Schroeder WS, Weirick BA, Hatler CW: Single Center Experience with OCR Guidance in the Treatment of Chronic Total Occlusions (CTO). Submitted to Transcatheter Cardiovascular Therapeutics; September 2001.

Cordero H, Underwood PL, Schroeder WS, Weirick BA, Hatler CW, **Heuser RR**: Fiber Optic Guidance Technology in the Treatment of Chronic Total Occlusions: Initial Experience. Presented at 12th Annual Symposium Transcatheter Cardiovascular Therapeutics; October 2000.

Laskey WK, **Heuser RR**, Suntharalingam M, Bass TA, Silber S, Rutherford BD, Wilmer C, Berke AD, Snell RJ, Popma J, Kuntz RE: Effects of ⁹⁰Sr/⁹⁰Beta Radiation on Diabetic Patients with In-Stent Restenosis. Presented at AHA November 2000.

Heuser RR, Cordero H, Underwood PL, Schroeder WS, Weirick BA, Hatler CW: OCR Can Be Used to Safely Treat Chronic Total Occlusions. Presented at AHA; November 2000.

Heuser RR, Cordero H, Underwood PL, Schroeder WS, Weirick BA, Hatler CW: Single Center Experience with OCR Guidance in the Treatment of Chronic Total Occlusions (CTO). Circulation October 23, 2001; Vol 104 (17) 1978.

Heuser RR, Cordero H, Schroeder W, Weirick BA, Hatler CW, Underwood PL: Fiberoptic Guidance System for Treatment of Chronic Total Occlusions: Single Center Experience. Presented at ACC 2001; March 2001.

Heuser RR, Lasky W, Bonan R, Lansky A: B-Radiation in Lesions Longer Than 20 Millimeters: A START 40/20 Subgroup Analysis. TCT 2001: September 2001.

Heuser RR, Underwood PL, Schroeder W, Weirick BA, Hatler CW: Experience with OCR Guidance in the Treatment of Chronic Total Occlusions. TCT 2001: September 2001.

PA Morales, **RR Heuser**, W Laskey, R Bonan, AJ Lansky, JJ Popma: Treating In-Stent Restenosis in Long Coronary Lesions. Presented at the 51st Annual Scientific Session American College of Cardiology; March 2002.

PA Morales, **RR Heuser**, EA Weirick, CW Hatler: Predicting Success in Crossing Chronic Total Occlusions With a New Guidewire. Presented at the 51st Annual Scientific Session American College of Cardiology; March 2002.

WK Laskey, M Suntharalingam, J Popma, **R Heuser**, B Rutherford, P Teirstein, A Lansky, R Kuntz, R Bonan: Efficacy of SR-90 Beta Radiation for the Treatment of In-Stent Restenosis: 24-Month Clinical Outcomes from the Stents And Radiation Therapy Trial (START). Presented at the 51st Annual Scientific Session American College of Cardiology; March 2002.

P.A. Morales, A. Reese, W. Schroeder, **R.R. Heuser**, New Treatment for Chronic Total Occlusions of Femoral Arteries. Presented at the Fourteenth Annual Symposium Transcatheter Cardiovascular Therapeutics; September, 2002

AWARDS & HONORS:

Columbia/HCA Cardiovascular Management Network - 1998 Cardiologist of the Year

PATENTS:

1. Method and Apparatus for Treating Body Tissues and Bodily Fluids; Patent granted December 12, 2000 Number: 6,159,197
2. Hot Tip Catheter; Patent granted February 20, 2001 Number: 6,190,379
3. Embolism Prevention Device; Patent granted April 2, 2002 Number: 6,364,900
4. Catheter apparatus and Method for Arterializing a Vein; Patent granted October 15, 2002 Number 6,464,665
5. Methods and apparatus for treating body tissues and bodily fluid vessels; Patent granted October 15, 2002 Number: 6,464,681
6. Catheter for Thermal Evaluation of Arteriosclerotic Plaque; Patent granted March 25, 2003 Number: 6,536,949
7. Small Diameter Snare; Patent granted April 29, 2003 Number: 6,554,842

**EXHIBIT
B**

DISCLOSURES

**APPLICATION
SERIAL NO. 09/064,000**

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

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Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Growth factor: a substance (as a vitamin B₁₂ or an interleukin)
that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D
CLAIMS
APPLICATION SERIAL NO. 09/064,000

382. A method for producing a desired soft tissue in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud in said body of said human patient; and
 - (c) Growing said desired soft tissue from said bud.
383. The method of claim 382, wherein said cells are multifactorial and non-specific.
384. The method of claim 383, wherein said cells comprise stem cells.
385. The method of claim 382 further comprising forming a new artery.
386. The method of claim 383 further comprising forming a new artery.
387. The method of claim 382, wherein said soft tissue comprises mesodermal tissue.
388. The method of claim 382, wherein said soft tissue comprises an artery.

EXHIBIT E

PUBLICATIONS

Clinical Investigation and Reports

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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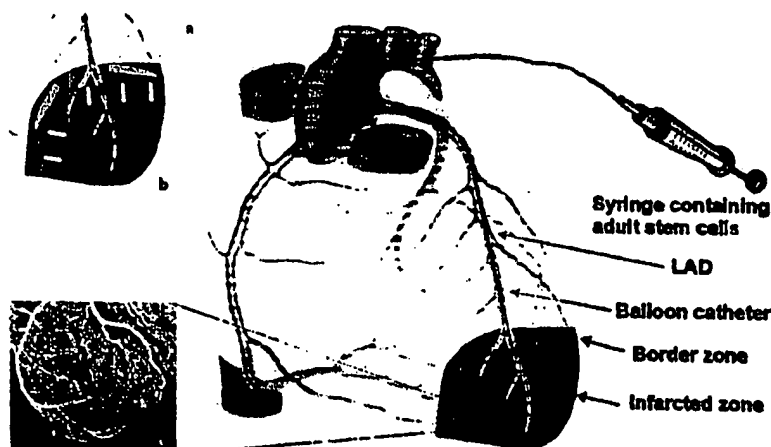


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery ($n=4$), left circumflex coronary artery ($n=3$), or right coronary artery ($n=13$). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty ($n=20$) and subsequent stent implantation ($n=19$). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients ($n=10$). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93\pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^5 ; this consisted of $0.65\pm 0.4\%$ AC133-positive cells and $2.1\pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radiolabeled ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ⁶)	2.8±2.2

Values are mean±SD or number of patients.

NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index P_{100}/ESV was calculated by dividing LV systolic pressure (P_{100}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11–14,18,20–23} Moreover, trans-endothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24–26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells ($\leq 1\%$), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

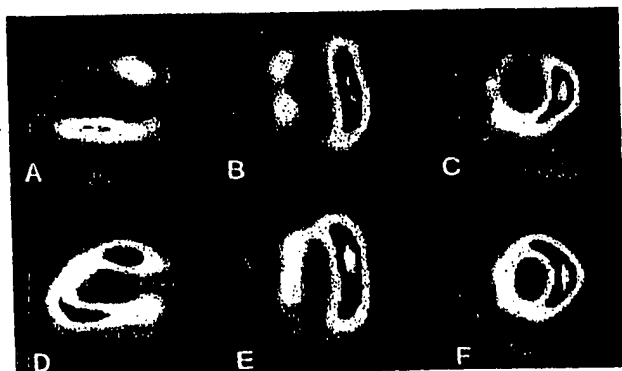


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ²⁰¹thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m ²	49±7	58±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P _{avg} /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
²⁰¹ Thallium scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells¹³; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of *in vitro* amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility (P_{100}/ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

References

- Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction: experimental observations and clinical implications. *Circulation*. 1990;81:1161-1172.
- Ertl G, Gaudron P, Hu K. Ventricular remodeling after myocardial infarction: experimental and clinical studies. *Basic Res Cardiol*. 1993;88:125-137.
- Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. *N Engl J Med*. 2002;346:5-15.
- Leor J, Patterson M, Quinones MJ, et al. Transplantation of fetal myocardial tissue into infarcted myocardium of rat: a potential method for repair of infarcted myocardium? *Circulation*. 1996;94(suppl II):332-336.
- Murry CE, Wiseman RW, Schwartz SM, et al. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest*. 1996;98:2512-2523.
- Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med*. 1998;4:929-933.
- Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation*. 1999;100(suppl II):247-256.
- Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? *Cell*. 2001;105:829-841.
- Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;105:369-377.
- Goodell MA, Jackson KA, Majka SM, et al. Stem cell plasticity in muscle and bone marrow. *Ann NY Acad Sci*. 2001;938:208-218.
- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-705.
- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA*. 2001;98:10344-10349.
- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430-436.
- Tomita S, Mickle DA, Weisel RD, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg*. 2002;123:1132-1135.
- Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. *Lancet*. 2001;357:279-280.
- Hamano K, Nishida M, Hirata K, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J*. 2001;65:845-847.
- Strauer BE, Brehm M, Zeus T, et al. Myocardial regeneration after intracoronary transplantation of human autologous stem cells following acute myocardial infarction. *Disch med Wschr*. 2001;126:932-938.
- Wang JS, Shum-Tim D, Chedrawy E, et al. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg*. 2001;122:699-705.
- Sheehan FH, Bolson EL, Dodge HT, et al. Advantages and applications of the Centerline method for characterizing regional ventricular function. *Circulation*. 1986;74:293-305.
- Sussman M. Cardiovascular biology: hearts and bones. *Nature*. 2001;410:640-641.
- Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-98.
- Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046-1052.
- Ferrari G, Cusella-De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*. 1998;279:1528-1530.
- Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634-637.
- Robinson SW, Cho PW, Levitsky HI, et al. Arterial delivery of genetically labeled skeletal myoblasts to the murine heart: long-term survival and phenotypic modification of implanted myoblasts. *Cell Transplant*. 1996;5:77-91.
- Bitner RE, Schofer C, Weipoltshammer K, et al. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat Embryol (Berl)*. 1999;199:391-396.
- Jiang Y, Jahagirdar B, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;20:1-12.
- Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood*. 2001;98:2615-2625.
- Condorelli G, Borello U, De Angelis L, et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc Natl Acad Sci USA*. 2001;98:10733-10738.
- Szilyassy SJ, Bass MJ, Van Zant G, et al. Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood*. 1999;93:1557-1566.
- Gregg DE, Fisher LC. Blood supply to the heart. In: *Handbook of Physiology*. Vol 2. Washington, DC: American Physiological Society; 1963:chap 44, 1517-1584.
- Strauer BE. Myocardial oxygen consumption in chronic heart disease: role of wall stress, hypertrophy and coronary reserve. *Am J Cardiol*. 1979;43:730-740.
- Toma C, Pittenger MF, Byrne BJ, et al. Adult human mesenchymal stem cells differentiate to a striated muscle phenotype following arterial delivery to the murine heart. *Circulation*. 2000;102(suppl II):II-683. Abstract.
- Hendriks PJ, Martens CM, Hagenbeek A, et al. Homing of fluorescently labeled murine hematopoietic stem cells. *Exp Hematol*. 1996;24:129-140.
- Ren G, Michael LH, Entman ML, et al. Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem*. 2002;50:71-79.
- Li RK, Mickle DA, Weisel RD, et al. Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. *Ann Thorac Surg*. 2001;72:1957-1963.
- Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002;53:31-47.
- Allgöwer M. *The Cellular Basis of Wound Repair*. Springfield, Ill: Charles C. Thomas; 1956.
- Xie Y, Zhou T, Shen W, et al. Soluble cell adhesion molecules in patients with acute coronary syndrome. *Chin Med J*. 2000;113:286-288.
- Soeki T, Tamura Y, Shinohara H, et al. Serial changes in serum VEGF and HGF in patients with acute myocardial infarction. *Cardiology*. 2000;93:168-174.
- Sheiban I, Frangasso G, Rosano GMC, et al. Time course and determinants of left ventricular function recovery after primary angioplasty in patients with acute myocardial infarction. *J Am Coll Cardiol*. 2001;38:464-471.

EVIDENCE APPENDIX

ITEM NO. 4

Supplemental Declaration of Dr. Heuser filed June 20, 2005



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

SUPPLEMENTAL DECLARATION OF RICHARD HEUSER, M.D.

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. This Supplemental Declaration is submitted in addition to my previous Declaration, dated November 11, 2004. No changes are made to my previous Declaration.
3. My Curriculum Vitae is attached as Exhibit A to my Declaration of November 11, 2004.
4. It is my understanding that the Examiner in charge of the above-identified patent application is also in the Examiner in charge of co-pending patent application Serial No. 09/794,456. In an Advisory Action dated November 26, 2004, for aforesaid Serial No. 09/794456, the Examiner questioned my qualification to render my opinions in my previous Declarations filed in such application. It is my further understanding that the Examiner reviewed my U.S. Patent No.

6,190,379 and did not find mention of delivery of any substance to the myocardium nor the word "cell." Also, the Examiner questioned my role in the cell delivery portion of Bioheart's laboratory and clinical trials using skeletal muscle cultured and modified. For the instant application, I provide the following information to respond to the Examiner's questions.

5. Regarding, U.S. Patent No. 6,190,379, the following is stated in Paragraph 3 of my Declaration:

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

By the above statement, I meant that the device shown in the patent has been used for the delivery of protein and/or muscle cells to the myocardium. At a presentation at the Angiogenesis Meeting in 1999 in Washington, D.C., we described this use of growth factors in a pig model with the development of neo vascularization. Moreover, I have had discussions with Bioheart regarding the use of my U.S. Patent No. 6,190,379 for delivery of cells.

Regarding my work at Bioheart, the following is stated in my Declaration:

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

To provide further information regarding the Examiner's questioning my involvement with Bioheart, I am a Scientific Advisory Board Member and in such role advise Bioheart throughout its pre-clinical and clinical work involving the

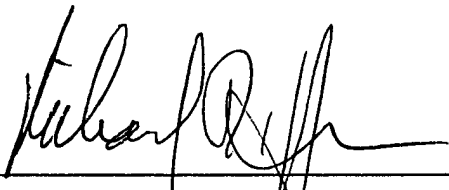
delivery of skeletal muscle cells into the myocardium. I am also an investigator with Bioheart's Phase 3 clinical trials in the United States. Such trials have not yet commenced.

6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/15/05



Richard Heuser, M.D., F.A.C.C., F.A.C.P.

EVIDENCE APPENDIX

ITEM NO. 5

**Second Supplemental Declaration of Dr. Heuser
cited by Appellant as Exhibit C in the Response filed June 26, 2006**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

SECOND SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I Richard Heuser declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.
2. My Curriculum Vitae is attached as Exhibit A to my Declaration of November 11, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my previous Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Second Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Second Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.
7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

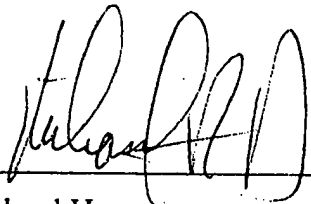
8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

6/19/06



Richard Heuser

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

NOVEMBER 1964
MEMORANDUM

TO : DIRECTOR

FROM : SAC, NEW YORK

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 382 A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud at said selected site in said body of said human patient; and
 - (c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
- Claim 383 The method of claim 382, wherein said cells are multifactorial and non-specific.
- Claim 384 The method of claim 383, wherein said cells comprise stem cells.
- Claim 385 The method of claim 382 further comprising forming a new artery.
- Claim 386 The method of claim 383 further comprising forming a new artery.
- Claim 387 The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

- Claim 388 The method of claim 382, wherein said soft tissue comprises an artery.
- Claim 389 The method of claim 382, wherein said cells comprise stem cells.
- Claim 390 The method of claim 389, wherein said soft tissue comprises an artery.
- Claim 391 The method of claim 382, wherein said cells comprise pluripotent cells.
- Claim 392 The method of claim 391, wherein said soft tissue comprises an artery.
- Claim 393 The method of claim 391, wherein said cells comprise stem cells.
- Claim 394 The method of claim 393, wherein said stem cells are multifactorial and non-specific.
- Claim 395 The method of claim 382, wherein said cells are injected into said body.
- Claim 396 The method of claim 382, wherein said cells are locally placed into said body.
- Claim 397 The method of claim 396, wherein said cells comprise stem cells.
- Claim 398 The method of claim 396, wherein said cells are injected intramuscularly.
- Claim 399 The method of claim 397, wherein said stem cells are injected intramuscularly.

- Claim 400 The method of claim 388 further comprising determining blood flow through said new artery.
- Claim 401 The method of claim 388 further comprising observing said new artery.
- Claim 402 The method of claim 399, wherein said selected site comprises a leg of said patient.

EVIDENCE APPENDIX

ITEM NO. 6

**Third Supplemental Declaration of Dr. Heuser
cited by Appellant as Exhibit C in the Response filed April 30, 2007**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 11, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 49, lines 18-22; page 53, line 1 through page 56, line 25; and page 62, lines 1-10;. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

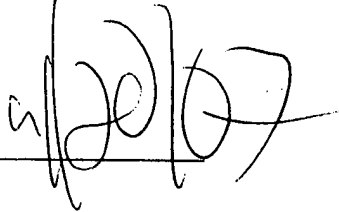
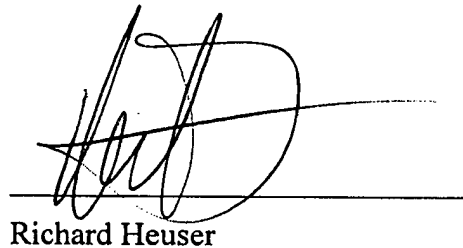
4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Third Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.
7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

A handwritten signature in black ink, appearing to be "12/20/07", written over a horizontal line.A handwritten signature in black ink, appearing to be "Richard Heuser", written over a horizontal line.
Richard Heuser

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT B

**ADDITIONAL
DISCLOSURES**

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 49, LINES 18-22

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEGF or BMP growth factors produced by VEGF or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 53, LINE 1 – PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution

comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1-10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT C

CLAIMS

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 382 A method for producing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud at said selected site in said body of said human patient; and
 - (c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
- Claim 383 The method of claim 382, wherein said cells are multifactorial and non-specific.
- Claim 384 The method of claim 383, wherein said cells comprise stem cells.
- Claim 385 The method of claim 382 further comprising forming a new artery.
- Claim 386 The method of claim 383 further comprising forming a new artery.
- Claim 387 The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

- Claim 388 The method of claim 382, wherein said soft tissue comprises an artery.
- Claim 389 The method of claim 382, wherein said cells comprise stem cells.
- Claim 390 The method of claim 389, wherein said soft tissue comprises an artery.
- Claim 391 The method of claim 382, wherein said cells comprise pluripotent cells.
- Claim 392 The method of claim 391, wherein said soft tissue comprises an artery.
- Claim 393 The method of claim 391, wherein said cells comprise stem cells.
- Claim 394 The method of claim 393, wherein said stem cells are multifactorial and non-specific.
- Claim 395 The method of claim 382, wherein said cells are injected into said body.
- Claim 396 The method of claim 382, wherein said cells are locally placed into said body.
- Claim 397 The method of claim 396, wherein said cells comprise stem cells.
- Claim 398 The method of claim 396, wherein said cells are injected intramuscularly.
- Claim 399 The method of claim 397, wherein said stem cells are injected intramuscularly.

- Claim 400 The method of claim 388 further comprising determining blood flow through said new artery.
- Claim 401 The method of claim 388 further comprising observing said new artery.
- Claim 402 The method of claim 399, wherein said selected site comprises a leg of said patient.
- Claim 403 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:
- (a) locally injecting stem cells into said body at said selected site;
 - (b) forming a bud at said selected site; and
 - (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.
- Claim 404 The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
- Claim 405 The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 406

The method of claim 402, wherein said desired soft tissue comprises an artery

EVIDENCE APPENDIX

ITEM NO. 7

**Fourth Supplemental Declaration of Dr. Heuser
cited by Appellant as Exhibit D
in the Response filed November 28, 2007**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**FOURTH SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 11, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures was attached to my Third Supplemental Declaration as Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 49, lines 18-22; page 53, line 1 through page 56, line 25; and page 62, lines 1-10;. A copy of such additional disclosures was attached to my Third Supplemental Declaration as Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit A and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Fourth Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.
7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit A without need for resorting to undue experimentation.

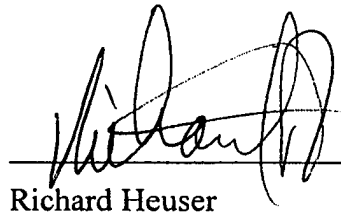
8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

11/21/07


Richard Heuser

FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT A

CLAIMS

EXHIBIT A
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 403 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:
- (a) locally injecting stem cells into said body at said selected site;
 - (b) forming a bud at said selected site; and
 - (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.
- Claim 404 The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
- Claim 405 The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.
- Claim 407 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

- Claim 408 The method of claim 407, wherein said bone marrow is from said patient.
- Claim 409 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.
- Claim 410 The method of claim 409, wherein said blood is from said patient.
- Claim 411 The method of claim 403 further comprising determining blood flow through said desired artery.
- Claim 412 The method of claim 403 further comprising observing said desired artery.

EVIDENCE APPENDIX

ITEM NO. 8

**Third Supplemental Declaration of Dr. Heuser
(originally filed in co-pending application Serial No. 10/179,589) and
cited by Appellant as Exhibit A in the Letter filed May 25, 2007**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 10/179,589)	
)	Examiner: Daniel C. Gamett
Filed: June 25, 2002)	
)	
For: METHOD FOR GROWING)	
HUMAN ORGANS AND)	
SUBORGANS)	

**THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.
3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Third Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20 line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Third Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Third Supplemental Declaration Exhibit C.
6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Third Supplemental Declaration Exhibit E. Note that in two of

the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Third Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

Richard Heuser

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT A

**MARCH 7, 2007
OFFICE ACTION
Paragraph 11, pages 7-9**

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6 , and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage as purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed to the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

Art Unit: 1647

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT C

**ADDITIONAL
DISCLOSURES**

EXHIBIT C
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1- 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEGF or BMP growth factors produced by VEGF or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8-17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT D

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6 , respectively.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT E

PUBLICATIONS (3)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (*From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London*)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under

therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu\text{g.} \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8.54	6.33	0.75
s.e. of obs.	2.89	3.03	0.326
Observed range	4.0-15.0	2.1-13.5	0.43-1.9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8.75	7.59	0.90
s.e. of obs.	3.05	3.72	0.30
Observed range	3.9-17.4	2.6-17.4	0.3-1.8

Table 2. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in $\mu\text{g.} \times 10^{-7}$ per cell

Group as a whole	Mean S.E. Observed range	DNAP 28 obs. on 12 cases	RNAP 10.9 5.03 2.3-25.1	Ratio DNAP/RNAP 28 obs. on 13 cases 0.87 0.27 0.35-1.5
		12.6 4.56 6.6-22.8		
Group prior to therapy	Mean	12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
	S.E.	12.57	13.38	1.06
	Observed range	4.17 8.1-22.8	5.19 7.5-25.1	0.249 0.69-1.5
Group during the course of therapy	Mean	17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
	S.E.	12.63	9.09	0.73
	Observed range	4.36 6.6-18.8	4.21 2.3-17.6	0.198 0.35-1.0

Table 3. *t test of significance between means*

	P	DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	Degrees of freedom	44	44	46
	P	<0.001	<0.001	0.2-0.1
Megaloblastic series before therapy compared with normal	Degrees of freedom	28	29	30
	P	0.01-0.001	<0.001	0.01-0.001
Megaloblastic series during therapy compared with normal	Degrees of freedom	33	33	34
	P	0.01-0.001	0.05-0.02	0.8-0.7
Megaloblastic series before and during therapy compared	Degrees of freedom	27	24	28
	P	0.7-0.6	0.05-0.02	<0.001
		Not significant	Significant	Highly significant

REFERENCES

- Davidson, J. N., Lealie, I. & White, J. C. (1947). *Biochem. J.* 41, xxvi.
 Davidson, J. N., Lealie, I. & White, J. C. (1948). *J. Path. Bact.* 50, 1.
 Mirsky, A. E. & Ris, H. (1949). *Nature, Lond.*, 163, 666.
 Vendrely, R. & Vendrely, C. (1948). *Experientia*, 4, 434.
 Vendrely, R. & Vendrely, C. (1949). *Experientia*, 5, 327.

Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébeq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (1 mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. Rat heart aconitase

Time (min.)	...	Citric acid (μmol.)	
		0	60
Additions:			
<i>cis</i> -Aconitate (5 μmol.)		0.21	3.90
<i>cis</i> -Aconitate + 'active' fraction		0.08	3.96
Citrate (5 μmol.)		4.90	4.34
Citrate + 'active' fraction		5.27	4.38

Table 2. Pig heart isocitric dehydrogenase

	<i>E</i> _{540 mμ.} (max. value)
DL-isocitrate only	0.076-0.065
Same + 'active' fraction	0.075
Same + <i>p</i> -chloromercuribenzoic acid 1.33 × 10 ⁻³ M	0.004

Table 3. Pig heart oxalosuccinic decarboxylase
(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min.
at 13.5° C. Net values)

	CO ₂ (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

REFERENCES

- Adler, E., v. Euler, H., Günther, G. & Plass, M. (1939). *Biochem. J.* 33, 1028.
 Buffa, P. & Peters, R. A. (1949). *J. Physiol.* 110, 488.
 Buffa, P., Peters, R. A. & Wakelin, R. W. (1950). In course of publication.
 Johnson, W. A. (1939). *Biochem. J.* 33, 1046.
 Liébeq, C. & Peters, R. A. (1949). *Biochim. Biophys. Acta*, 3, 215.
 Martius, C. (1949). *Liebigs Ann.* 561, 227.
 Ochoa, S. & Weiss-Tabori, E. (1948). *J. biol. Chem.* 174, 123.

Appendix

Nucleic Acids

Nucleic Acids

Content and Distribution

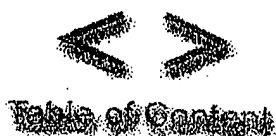
Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	$0.51.0 \times 10^5$
	1.5×10^4
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
	~14% of total RNA
Ratio of DNA:RNA in nucleus	~ 2:1
Number of mRNA molecules ^c	$0.2 \ 1.0 \times 10^6$
Number of different mRNA species	
Low abundance mRNA (5 15 copies/cell)	$1.0 \ 3.4 \times 10^4$
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
	<10 different messages
Abundance of each message for:	
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

- a 30 – 60 µg/ml blood for human leukocytes.
b 1 – 5 µg/ml blood for human leukocytes.
c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells)	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6



UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1 µg	1 nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA $\sim 3 \times 10^6$ nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (diploid)	0.03 pg
<i>E. coli</i>	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
<i>S. cerevisiae</i>	5 μm	66 μm^3
<i>S. pombe</i>	2 x 7 μm	22 μm^3
Mammalian cell	10-20 μm	500-4,000 μm^3
<i>E. coli</i>	1 x 3 μm	2 μm^3
Mammalian mitochondrion	1 μm	0.5 μm^3
Mammalian nucleus	5-10 μm	66-500 μm^3
Plant chloroplast	1 x 4 μm	3 μm^3
Bacteriophage lambda	50 nm (head only)	$6.6 \times 10^{-5} \mu\text{m}^3$
Ribosome	30 nm diameter	$1.4 \times 10^{-5} \mu\text{m}^3$
Globular monomeric protein	5 nm diameter	$6.6 \times 10^{-8} \mu\text{m}^3$

III. Some useful concentrations

Total cell protein concentration Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

Abundant
transcription
factor

Rare transcription
factor

1 nM (100,000 copies/ nucleus)
10 pM (1,000 copies/ nucleus)

Serum

50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	μg
10,000 Da protein	1

100,000 Da protein

110

Protein/ DNA conversions1 kb of DNA encodes 333 amino acids $\approx 3.7 \times 10^4$ Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10 -30 pg
Proportion of total RNA in nucleus	$\sim 14\%$
DNA:RNA in nucleus	$\sim 2:1$
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5 - 1×10^5
Active genes	1.5×10^4
mRNA molecules	2×10^5 - 1×10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (μ g)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocytes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O ₂ & CO ₂ transport
Cells per ml	4-7 x 10 ⁶	3-4 x 10 ⁸	5 x 10 ⁹
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNA content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 10⁶ per ml in cases of immunosuppression, to 40 x 10⁶ during inflammation, to 500 x 10⁶ during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

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Mit Urteil vom 12. Mai 1998 hat das Landgericht Hamburg entschieden, dass man durch die Ausbringung eines Links die Inhalte der gelinkten Seite ggf. mit zu verantworten hat. Dies kann - so das LG - nur dadurch verhindert werden, dass man sich ausdrücklich von diesen Inhalten distanziert. Wir haben auf verschiedenen Seiten dieser Homepage Links zu anderen Seiten im Internet gelegt. Für all diese Links gilt: "Wir möchten ausdrücklich betonen, dass wir keinerlei Einfluss auf die Gestaltung und die Inhalte der gelinkten Seiten haben. Deshalb distanzieren wir uns hiermit ausdrücklich von allen Inhalten der gelinkten Seiten auf der Website inklusive aller Unterseiten und machen uns ihre Inhalte nicht zu eigen." Diese Erklärung gilt für alle auf der Homepage ausgebrachten Links und für alle Inhalte der Seiten, zu denen Links führen.

EVIDENCE APPENDIX

ITEM NO. 9

**Declaration of Dr. Andrew E. Lorincz cited by Appellant as an Exhibit
in the Amendment filed February 15, 2001**



IN THE UNITED STATES PATENT AND TRADE-MARK OFFICE

APPLICANT: James P. Elia)
SERIAL NO.: 09/064,000) EXAMINER: Nicholas D. Lucchesi
FILED: April 21, 1998) GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS)
FOR INSTALLATION OF)
DENTAL IMPLANT)

DECLARATION OF ANDREW E. LORINCZ, M.D.

I Andrew E. Lorincz declare as follows:

1. I reside at 3628 Belle Meade Way, Mountain Brook, Alabama 35223.
2. My Curriculum Vitae is attached hereto as Exhibit A.
3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.
4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2-12-01

Andrew E. Lorincz
Andrew E. Lorincz

C:\MYDOCUMENTS\CLIENTS\SUBJ\AW\ORINC\DECLARATION.DOC

EXHIBIT A

CURRICULUM VITAE

NAME: Andrew E. Lorincz, M.D.
TITLE: Professor of Pediatrics
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Telephone: (205) 967-4678

BIRTH: 5/17/26 Chicago, Illinois

MARITAL STATUS: Married, 12/14/65 - Diane DeNyse Lorincz

EDUCATION:

1948-1952 University of Chicago, School of Medicine, M.D. Degree
1948-1950 University of Chicago, B.S. Degree (Anatomy & Biochemistry)
1946-1948 University of Chicago, Ph.B. Degree

POSTDOCTORAL EDUCATION:

Jan-Mar 1980 Lysosomal Storage Disease Laboratory, Eunice Kennedy Shriver Center,
Waltham, MA (Harvard), Visiting Scientist
1955-1956 LaRabida Jackson Park Sanitarium, University of Chicago,
Junior Staff Physician Department of Pediatrics, University of Chicago
Clinics Bob Roberts Memorial Hospital
1955-1958 Arthritis and Rheumatism Foundation Fellow
1954-1955 Benjamin J. Rosenthal Clinical and Research Fellow
1953-1954 Junior Assistant Resident
1952-1953 Intern

ACADEMIC APPOINTMENTS:

1996-present	Professor Emeritus, Department of Pediatrics
1984-1996	School of Public Health, University of Alabama at Birmingham, Professor
1971-1996	University of Alabama at Birmingham, Member of Graduate Faculty
1968-1996	University of Alabama at Birmingham, Professor of Pediatrics
1971-1984	Division of Engineering Biophysics, University of Alabama at Birmingham, Associate Professor
1968-1982	University of Alabama at Birmingham, Associate Professor of Biochemistry
1976-1980	School of Optometry, University of Alabama at Birmingham, Professor Optometry
1971-1980	School of Nursing, University of Alabama at Birmingham, Clinical Associate Professor
1970-1980	Center for Developmental and Learning Disorders, University of Alabama at Birmingham, (A University Affiliated Facility for Developmental Disability), Director
1970-1976	School of Optometry, University of Alabama at Birmingham, Associate Professor of Pediatric Optometry
1966-1968	Medical Teaching and Research, Unit of the University of Florida at the Sunland Training Center, Gainesville, Florida, Director
1963-1968	Department of Surgery (Orthopaedics), University of Florida College of Medicine, Gainesville, Florida, Research Associate Professor
1962-1968	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Associate Professor
1959-1962	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Assistant Professor
1956-1959	Department of Pediatrics, University of Chicago School of Medicine, Chicago, Illinois, Instructor

PROFESSIONAL LICENSES - PHYSICIAN AND SURGEON:

5/26/69	State of Alabama
8/10/59	State of Florida (inactive)
9/22/54	State of Illinois (inactive)

SPECIALTY CERTIFICATION:

May 1958 American Board of Pediatrics, Diplomate

BOARDS, COMMITTEES AND CONSULTANTSHIPS:

1994-present	Board member of The Mental Retardation and Developmental Disabilities, Health Care Authority of Jefferson County, Inc.
1991-present	Editorial Board for the <u>Annals of Clinical and Laboratory Science</u> , Member
1988-present	Medical Advisory Board of the National MPS Society, Member
1980-1986	<u>Mental Retardation</u> , Consulting Editor
1979-present	National Tay-Sachs and Allied Diseases Association, Scientific Advisory Committee, Member
1978-present	Mayor's Council of Disability Issues
1979-1984	Osteogenesis Imperfecta Foundation, Inc., Board Member Alabama O.I. Chapter
1974-1981	Child Mental Health Services, Inc., Birmingham, Alabama, Board Member
1977-1978	Elizabethtown Committee on Planning and Evaluation, Legislative Committee, State of Pennsylvania
1973-1975	Human Rights Committee for the Partlow State School and Hospital, Tuscaloosa, Alabama, Member - Federal Court Appointed
1971-1974	American Academy of Pediatrics, Committee on Children With Handicaps
1971-1973	<u>American Journal of Mental Deficiency</u> , Consulting Editor
1965-1973	Head Start, Medical Consultant
1967-1972	<u>Journal of Investigative Dermatology</u> , Editorial Consultant
1961-1968	Sunland Hospital, Orlando, Florida, Medical and Research Consultant
1965-1966	State of Florida Interagency Committee on Mental Retardation Planning, Co-Chairman, Mental Retardation Research Committee <u>Alabama Developmental Disabilities Planning Council</u>
1982-1984	Maternal and Child Health, Member of Advisory Committee
1979-1984	Member (Secretary, 1980; Vice Chairman, 1984)
1973-1979	Consultant

American Association of University Affiliated Facilities

1975-1978 American Association of University Affiliated Programs for the Developmentally Disabled, Board Member

American Association on Mental Retardation

1980,84,85	Prevention Committee, Chairman
1980-1982	Member of Council
1978-1980	Medicine Division and Member Executive Committee, Vice President

BOARDS, COMMITTEES AND CONSULTANTSHIPS: (CONTD)

Association of Retarded Citizens of Jefferson County

1990-present	Board Member
1975-1985	Board Member
1977-1978	Second Vice President
1980	Recipient of Distinguished Service Award

PROFESSIONAL SOCIETIES:

American Academy for Cerebral Palsy and Developmental Medicine, (Fellow)
American Academy on Mental Retardation (President Elect, 1975-76; President, 1976-77)
Emeritus Member
American Academy of Pediatrics (Fellow)
American Association for the Advancement of Science
American Association for Clinical Chemistry, Inc.
American Association on Mental Retardation (Fellow)-Life Member
American Chemical Society
American Federation for Clinical Research
American Medical Association
American Society for Human Genetics
American Society for Investigative Pathology
Association of Clinical Scientists
International Society for Mycoplasmaology
Jefferson County Pediatric Society
Society for Complex Carbohydrates
Society for Investigative Dermatology
Society for Pediatric Research
Society for Sigma Xi
Southern Society for Pediatric Research (President, 1964)

BIBLIOGRAPHY

1. Dorfman, A.; Lorincz, A.E.: Occurrence of Urinary Acid Mucopolysaccharides in the Hurler Syndrome. Proceedings of the National Academy of Science, 43:443-46, 1957.
2. Dorfman, A., Gross, J.I.; Lorincz, A.E.: The Treatment of Acute Rheumatic Fever. Pediatrics, 27:692-705, May, 1961.
3. Lorincz, A.E.: Heritable Disorders of Acid Mucopolysaccharide Metabolism in Humans and in Shorter Dwarf Cattle. Annals of the New York Academy of Science, 91:644-58, 1961.
4. Shepard, T.H., Lorincz, A.E., Gartner, S.M.: Desulfuration of Thiourea by Saliva. Proceedings of the Society of Experimental Biology and Medicine, 112:38-42, July, 1963.

BIBLIOGRAPHY (CONT'D)

5. Lorincz, A.E.: A Heritable Disorder of Acid Mucopolysaccharide Metabolism in Cattle Which Resembles the Hurler Syndrome. Proceedings of the Second International Congress on Mental Retardation, Vienna, 1961, Part I: 70-74, 1963.
6. Gennaro, J.F., Jr., Callahan, W.P., III, Lorincz, A.E.: The Anatomy and Biochemistry of a Mucus-secreting Cell Type Present in the Poison Apparatus of the Pit Viper, Ancistrodon Piscivorus Piscivorus. Annals of the New York Academy of Sciences, 106:463-71, March, 1963.
7. Wolfson, S.L., Davidson, E., Harris, J.S., Kahana, L., Lorincz, A.E.: Long-term Corticosteroid Therapy in Hurler Syndrome. American Journal of Diseases in Children, 106:3-10, July, 1963.
8. Wainer, A., Lorincz, A.E.: Studies of Mercapturic Acid Synthesis by Humans. Life Sciences, No. 7:504-08, 1963.
9. Lorincz, A.E.: Hurler's Syndrome in Man and Snorter Dwarfism in Cattle: Heritable Disorders of Connective Tissue Acid mucopolysaccharide Metabolism. Clinical Orthopaedics and Related Research, A.F. DePalma (Editor), J.B. Lippincott Co., 33:104-18, 1964.
10. Pearson, H.A., Lorincz, A.E.: A Characteristic Bone Marrow Finding in the Hurler Syndrome. Pediatrics, 34:281-82, August, 1964.
11. Brodgon, B.G., Bartley, T.D., Schiebler, G.L., Shanklin, D.R., Krovetz, I.J., Lorincz, A.E.: Cardiovascular Radiology in Calves. Angiology, 15:496-504, November, 1964.
12. Lorincz, A.E.: Hurler's Syndrome. Medical Aspects of Mental Retardation, C.H. Carter (Editor), Chapter 19, pp. 628-650, 1965.
13. Krovetz, I.J., Lorincz, A.E., Schiebler, G.L.: Cardiovascular Manifestations in the Hurler Syndrome. Hemodynamic and Angiocardiographic Observations in 15 Patients. Circulation, 31:132-141, 1965.
14. Gennaro, J.F., Jr., Lorincz, A.E., Brewster, H.B.: The Anterior Salivary Gland of the Octopus (Octopus Vulgaris) and its Mucous Secretion. Annals of the New York Academy of Science, 118:1021-25, November, 1965.
15. Gessner, I.H., Lorincz, A.E., Bostrom, H.: Acid Mucopolysaccharide Content of the Cardiac Jelly of the Chick Embryo. Journal of Experimental Zoology, 160:291-98, No. 3, December, 1965.
16. Callahan, W.P., Lorincz, A.E.: Hepatic Ultrastructure in the Hurler Syndrome. American Journal of Pathology, 48:277-98, No. 2, February, 1966.
17. Callahan, W.P., Hackett, R.L., Lorincz, A.E.: New Observations by Light Microscopy on Liver Histology in the Hurler's Syndrome. Archives of Pathology, 83:507-12, June, 1967.
18. Lorincz, A.E.: Screening Measurements of Urinary Acid Mucopolysaccharides for Detection of the Hurler Syndrome. The Clinical Pathology of Infancy, Sunderman, F.W. & Sunderman, F.W., Jr. (Editors), pp. 68-71, 1967.
19. Rhoades, R., Lorincz, A.E., Gennaro, J.F., Jr.: Polysaccharide Content of the Poison Apparatus of the Cottonmouth Moccasin, Akistrodon Piscivorus Piscivorus. Toxicon, 5:125-31, 1967.

BIBLIOGRAPHY (CONT'D)

20. Krovetz, L.J., Lorincz, A.E., Schiebler, G.L.: Cardiovascular Manifestations of the Hunter-Hurler Syndrome. Heart Diseases in Infants, Children and Adolescents, A.J. Moss & F.H. Adams (Editors), The Williams and Wilkins Co. (Publisher), 1968.
21. Lorincz, A.E.: Hurler's Syndrome - Disorders in Systematic Disease - Disorders of the Mucopolysaccharides. The Growth Plate and Its Disorders, M. Rang (Editor), E & S Livingston, Ltd., London, pp. 49-56, 1969.
22. Lorincz, A.E.: Pediatric Dermatology. Dermatology in General Medicine, Fitzpatrick, Thomas, B., arndt, Kenneth, A., Clark, Wallace, H., Eisen, Authur, Z., Van Scott, Eugens, J., Vaughn (Editors), John H. McGraw-Hill, Blakiston Division, N.Y., pp. 1999-2006, 1971.
23. West, S.S., Lorincz, A.E.: Fluorescent Molecular Probes in Fluorescence Microspectrophotometry and Microspectropolarimetry. Fluorescence Techniques in Cell Biology, A.A. Thacr & M. Sernetz (Editors), Springer-Verlag, Berlin-New York-Heidelberg, pp. 395-407, 1973.
24. McMahon, J., Kugel, R.B., Bartram, J.H., Berenberg, W., Cavanaugh, J.J., Hanson, V., Kennell, J.H., Lorincz, A.E., Pearson, P.H., Scott, R.B., Scurletis, T.D., Taft, L.F. (Committee on Children With Handicaps, american Academy of Pediatrics): The Physician and the Deaf child. Pediatrics, 51:1100-01, No. 6, June, 1973.
25. Thompson, J.N., Finley, S.C., Lorincz, A.E., Finley, W.H.: Absence of -L-Iduronidase Activity in Various Tissues from Two Sibs Affected Presumably With Hurler-Scheie Compound Syndrome. Birth Defects: Original Article Series, Disorders of Connective Tissues, XI: 341-46, No. 6, 1975.
26. Hurst, R.E., Cezayirli, R.C., Lorincz, A.E.: Nature of the Glycosaminoglycanuria (Mucopolysacchariduria) in Brachycephalic "Shorter" Dwarf Cattle. Journal of Comparative Pathology, 85:481-86, 1975.
27. Lorincz, A.E., Montes, L.F.: Mucopolysaccharidosis (Type I Hurler-Scheie Compound). Journal of Cutaneous Pathology, 2:214-15, 1975.
28. Hurst, R.E., Settine, J.M., Lorincz, A.E.: A Method for the Quantitative Determination of Urinary Glycosaminoglycans. Clinica Chimica Acta, 70:427-32, 1976.
29. Hurst, R.E., Jennings, G.C., Lorincz, A.E.: Partition Techniques for Isolation and Fractionation of Urinary Glycosaminoglycans. Analytical Biochemistry, 79:502-12, 1977.
30. Lorincz, A.E.: The Mucopolysaccharidoses: Advances in Understanding and Treatment. Pediatric Annals, 7:64-98, 1978.
31. Lorincz, A.E.: Mucopolysaccharidoses. Medical Aspects of Mental Retardation, Charles H. Carter (Editor), Charles C. Thomas (Publisher), 2nd edition, 1978.
32. Lorincz, A.E.: Perspectives on Planning for Prevention of Mental Retardation. Planning for Services to Handicapped Persons: Community, Education, & Health, Magrab, P.R. & Elder, J.O. (Editors), P.H. Brooks (Publisher), Chapter 3, pp. 77-90, 1979.
33. Hurst, R.E., Settine, J.M., Floyd, W.M., Lorincz, A.E.: Glycosaminoglycan Excretion in Osteogenesis Imperfecta. Clinica Chimica Acta, 100:307-11, 1980.

BIBLIOGRAPHY (CONT'D)

34. Lorincz, A.E.: Learning Disabilities - A Pediatrician's Perspective. The Role of Vision in the Multidisciplinary Approach to Children with Learning Disabilities, Morton Davis & John C. Whitener (Editors), Charles C. Thomas (Publisher), Springfield, Illinois, Chapter 2, pp. 18-40, 1982.
35. Lorincz, A.E., Hurst, R.E., Kolodny, E.H.: The Early Laboratory Diagnosis of Mucopolysaccharidoses. Annals of Clinical and Laboratory Science, 12:258-66, 1982.
36. Lorincz, A.E.: Glycosaminoglycans in Heritable and Developmental Disorders of Bone. Glycosaminoglycans and Proteoglycans in Physiological and Pathological Processes of Body Systems, R.S. Varma and R. Varma (Editors), S. Karger AG (Publisher), Switzerland, pp. 264-75, 1982.
37. Lorincz, A.E., Kolodny, E.H.: The Early Diagnosis of Mucopolysaccharidoses. Proceedings of the 6th Congress of the International Association for the Scientific study of Mental Deficiency, J.M. Berg (Editor), Volume II, 1983.
38. Lorincz, A.E.: Rapid Method for the Identification of Mycoplasma Organisms. Manual of Procedures for Applications of Nucleic Acid Probes and Monoclonal Antibodies in Human Disease, F. William Sunderman (Editor), Institute for Clinical Science, Inc. (Publisher), pp. 163-165, November 13-16, 1986.
39. Lorincz, A.E.: Rapid Fluorescence Technique for the Detection of Toxic Pulmonary Microorganisms, e.g. Legionella Pneumophila. Manual of Procedures for Clinical and Analytical Toxicology, F. William Sunderman (Editor), Institute for Clinical Science, Inc. (Publisher), pp. 129-131, November 12-15, 1987.
40. Reque, P.G., Lorincz, A.E.: Supravital Microscopic Fluorescent Technique for the Detection of Tinea capitis. Cutis, 42:111-114, August 1988.
41. Petcharuttana, Y., Cutter, G.R., Meeks, R.G., & Lorincz, A.E.: Fluorescence Microscopy of DFS-induced Morphologic Transformation in Unfixed, Cultured Cells. Journal of Oral Pathology and Medicine, 18:451-456, 1989.
42. Lorincz, A.E.: Supravital Microscopic Fluorescence Technique for the Detection of Malaria and Other Parasitic Organisms. Manual of Procedures on Laboratory Diagnosis of Diseases of the Liver, F. William Sunderman (Editor), Institute for Clinical Science, Inc. (Publisher), pp. 89-92, November 16-19, 1989.
43. Lorincz, A.E.: Rapid Screening for Fungal Organisms Utilizing Supravital Fluorescence Microscopy. Manual of Procedures on Laboratory Diagnosis of Metabolic Disorders With Emphasis on Diabetes Mellitus, F. William Sunderman (Editor), Institute for Clinical Science, Inc. (Publisher), pp. 191-193, November 12-15, 1992.
44. Lorincz, A.E., Kelly, D.R., Dobbins, G.C., Cardone, V.S., Fuchs, S.A., Schilleci, J.L.: Urinalysis: Current Status and Prospects for the Future. Annals of Clinical and Laboratory Science, Vol 29, No 23, pp.169-175

ABSTRACTS

1. Lorincz, A.E., Dorfman, A.: Occurrence of Urinary Acid Mucopolysaccharides in the Hurler Syndrome. American Journal of Diseases of Children, 94:552, June, 1957.
2. Lorincz, A.E.: Acid Mucopolysaccharides in the Hurler Syndrome. Federation Proceedings, 17:226, March, 1958.
3. Kroger, M., Warnick, A.C., Hentges, J.F., Lorincz, A.E.: Mucopolysaccharidosis in Snorter Dwarf Cattle. Journal of Animal Science, 19:1221, 1960.
4. Lorincz, A.E.: "Snorter" Dwarf Cattle: A Naturally Occurring Heretable Disorder of Acid Mucopolysaccharide Metabolism Which Resembles Hurler Syndrome. American Journal of Diseases of Children, 100:488, 1960.
5. Lorincz, A.E.: Urinary Acid Mucopolysaccharides in Hereditary Deforming Chondrodysplasia (Diaphysical Aclais). Federation Proceedings, 19:148, December, 1960.
6. Lorincz, A.E.: Urinary Acid Mucopolysaccharides in Hereditary Arthrodysplasia. Southern Medical Journal, 53:1588, March, 1960.
7. Wolfson, S.L., Kahana, L., Harris, J.S., Lorincz, A.E.: Biochemical and Roctgenographic Response to Long-term Corticosteroid Therapy on the Hurler Syndrome. American Journal of Diseases of Children, 102:638, 1961.
8. Lorincz, A.E.: Mucopolysacchariduria in Children With Hereditary Arthro-osteo-onychodysplasia. Federation Proceedings, 21:173, March/April, 1962.
9. Schiebler, G.L., Lorincz, A.E., Brodgon, B.G., Shanklin, D.R., Bartley, T.D., Krovetz, L.J.: Cardiovascular Manifestations of Hurler's Syndrome. Circulation, 26:782, 1962.
10. Gessner, L.H., Lorincz, A.E.: Acid Mucopolysaccharide Content of the Three-day Old Chick Embryo Heart. Federation Proceedings, 22:2, December, 1963.
11. Krovetz, L.J., Lorincz, A.E., Schiebler, G.L.: Hemodynamic Studies in the Hunter-Hurler Syndrome (Gargoylism). Circulation, 28:753., 1963.
12. Batey, R.L., Lorincz, A.E.: A New Technique for the Identification of Acid Mucopolysaccharide Hydrolysates. Southern Medical Journal, 56:1437, 1963.
13. Callahan, W.P., Lorincz, A.E.: Changes in Liver Ultrastructure Associated With Hurler Syndrome. Anatomical Record, 148:267, 1964.
14. Renn, W.H., Lorincz, A.E.: A New Urinary Mucopolysaccharide Associated With the Nail-Patella Syndrome (Hereditary arthro-osteo-onychodydysplasia). Southern Medical Journal, 58:1584, December, 1965.
15. Lorincz, A.E., Callahan, W.P.: Electron Microscopic Observations of Liver in the Hurler Syndrome. Southern Medical Journal, 58:1584, December, 1965.
16. Renn, W.H., Enneking, W.F., Hase, M.F., Lorincz, A.E.: Acid Mucopolysaccharide Content of Human Chondrosarcomatous Tumors. Journal of Bone and Joint Surgery, 50-A:1072, 1968.
17. Lorincz, A.E., Tiller, R.E., West, S.S.: A New Biophysical Cytochemical Finding in Viable Leukocytes Obtained from Patients with Cystic Fibrosis. Cystic Fibrosis Club Abstracts, 13th Annual Meeting, May 20, 1972.
18. Lorincz, A.E., Finley, S.C., Finley, W.H., West, S.S.: Biophysical Cytochemical Study of Glycosaminoglycans in Fibroblasts and Other Cells Derived From Marteaux-Lamy Mucopolysaccharidosis. Federation Proceedings, Vol. 31, No. 2, March-April, 1973.

ABSTRACTS (CONT'D)

19. Warren, R.J., Condrón, C.J., Hollister, D., Huifing, F., Neufeld, E.F., Hall, C.W., Mcleod, A.G.W., Lorincz, A.E.: Antenatal Diagnosis of Mucopolidosis II (I-Cell Disease). Pediatric Research, Vol. 7, No. 343, April, 1973.
20. Lorincz, A.E., West, S.S.: The Biophysical Cytochemistry of Living Cells Derived From Patients With Mucopolysaccharidoses. Journal of Bone and Joint Surgery. Proceedings of the 19th Annual Meeting of the Orthopedic Research Society, Las Vegas, Nevada, 55-A:655, January 30, 31 & February 1, 1973.
21. Hurst, R.E., Cezayirli, R.C., Lorincz, A.E.: The Nature of the Glycosaminoglycans (Mucopolysacchariduria) in Brachycephalic "Snorter" Dwarf Cattle. Federation Proceedings, 34:861, March, 1975.
22. Lorincz, A.E., Hurst, R.E., Cezayirli, R.C.: Definitive Characterization of the Glycosaminoglycanuria in "Snorter" Dwarf Cattle. Pediatric Research, 9:314, 1975.
23. Lorincz, A.E., Hurst, R.E., Floyd, W.M.: Glycosaminoglycan excretion of Osteogenesis Imperfecta. Transactions of the 25th Annual Meeting. Orthopedic Research Society. Journal of Bone and Joint Surgery, 4:108, 1978.
24. Lorincz, A.E.: Clinical Chemistry in the Profoundly Retarded. Annals of Clinical and Laboratory Science, 14:316-17, 1984.
25. Lorincz, A.E.: Direct Visualization of Mycoplasma via Supravital Staining and Fluorescence Microscopy. Abstracts of the 6th International Congress of the International Organization for Mycoplasma, p. 272, 1986.
26. Lorincz, A.E., Thompson, J.N.: Use of Microscopic Fluorescence Amniotic Fluid to Diagnose Intrauterine Mucopolysaccharidosis. 1st International Congress on Mucopolysaccharidosis and Related Diseases, p. 33, 1988.
27. Lorincz, A.E., Reque, P.G.: Comparison of Supravital Microscopic Fluorescence Technique (SMFT) to Darkfield Microscopy for Detection of Spirochetes. Annals of Clinical and Laboratory Science, 20:281-82, 1990.
28. Lorincz, A.E.: One Step On-site Epi-fluorescence Detection of Fungi: A Possible Alternate to KOH Screening. Annals of Clinical and Laboratory Science, 23: 307, 1993.
29. Lorincz, A.E. and Bueschen, A.J.: Mycoplasma-like Mollicutes in Human Prostate Tumor Tissue. Annals of Clinical and Laboratory Science, 24: 196, 1994.
30. Lorincz, A.E., Baltaro, R.J. and Adamson, D.M.: Detection of Significant Bacteruria Using Supravital Fluorescence Microscopy. Annals of Clinical and Laboratory Science, 25: 363, 1995.
31. Lorincz, A.E., Bueschen, A.J., and Urban, D.A.: Presence of Mycoplasma-Like Mollicutes In Prostate Needle Biopsy Specimens, Annals of Clinical and Laboratory Science, 26:364, 1996.

EDITORIALS AND COMMENTARIES

1. Lorincz, A.E.: Biochemical Genetic Defects. Journal of the Florida Medical Association, 66:852, 1960.
2. Lorincz, A.E., Smith, R.T.: Research at the University of Florida College of Medicine, Department of Pediatrics. Journal of the Florida Medical Association, 51:808-11, 1965.
3. Lorincz, A.E.: Birth Defects - A Continuing Challenge. Journal of the Florida Medical Association, 55:126-27, 1968.

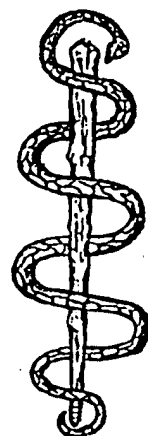
BOOK REVIEWS

1. "Modern Topics in Paediatric Dermatology", Verbov, J. (Editor), J.B. Lippincott, Philadelphia (Publisher), JAMA, 244:1269, 1980.
2. "Dermatologic Disorders in Black Children and Adolescents", Laude, T.A. & Russo, R.U. (Editors), Medical Examination Publishing Company, New Hyde Park, New York (Publisher), JAMA, 252:1769-70, 1984.
3. "Practical Pediatric Dermatology", Weston, W.L. (Editor), Little Brown and Company, Boston (Publisher), JAMA, 277:549, 1987.

EXHIBIT B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY



ILLUSTRATED

*A vocabulary of medicine and
its allied sciences, with pronunciations
and derivations*

TWENTY-SECOND EDITION

*Completely revised by a staff of 33 editors, covering
44 specialties and subspecialties*

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BALTIMORE



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Publisher's

How to Get

Pronunci

Guide to

Abbrevia

Spelling.

Organiza

Main

Alph

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Alph

Cros

Special C

Anat

Chem

Epon

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Medical Ety

Word For

Direction

Root Wor

Greek and

Plural, Ad

Vocabulary .

Appendices

1A. Pharr

1B. Snake

2. Blood

3. Gloss

4. Proof

5. Weigl

6. Symb

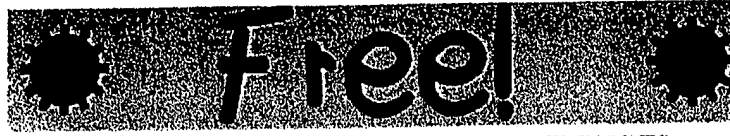
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10. Gloss

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<input type="text" value="bud[2,verb]"/>	<input type="button" value="Go To"/>
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Main Entry: ¹**bud****Pronunciation:** 'b&d**Function:** *noun***Etymology:** Middle English *budde***Date:** 14th century**1 :** a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot**2 :** something not yet mature or at full development: as **a :** an incompletely opened flower **b :** CHILD, YOUTH **c :** an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM**- in the bud :** in an early stage of development in the bud>Dictionary Look Up: Type in your word or phrase and click Search. Click on HELP for search tips.

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* generally or often considered vulgar

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Thesaurus

Main Entry: **pri·mor·di·um**

Pronunciation: -dE-&m

Function: *noun*

Inflected Form(s): *plural pri·mor·dia* /-dE-&/

Etymology: New Latin, from Latin

Date: 1671

: the rudiment or commencement of a part or organ

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Thesaurus Symbol Key

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Dictionary Pronunciation Key

- | | | |
|--------------------------------|------------------------|------------------------|
| • \&\ as a and u in abut | • \e\ as e in bet | • \o\ as aw in law |
| • \&\ as e in kitten | • \E\ as ea in easy | • \oi\ as oy in boy |
| • \&r\ as ur and er in further | • \g\ as g in go | • \th\ as th in thin |
| • \a\ as a in ash | • \i\ as i in hit | • \th\ as th in the |
| • \A\ as a in ace | • \I\ as i in ice | • \ü\ as oo in loot |
| • \ä\ as o in mop | • \j\ as j in job | • \u\ as oo in foot |
| • \au\ as ou in out | • \[ng]\ as ng in sing | • \y\ as y in yet |
| • \ch\ as ch in chin | • \O\ as o in go | • \zh\ as si in vision |



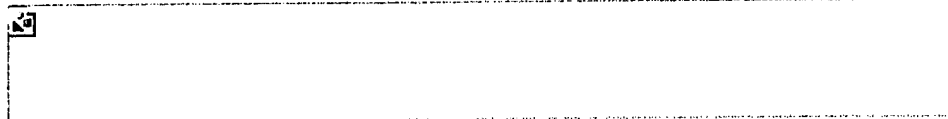
Encyclopædia Britannica

organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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Thesaurus	organ
	barrel organ
Go To	electric organ

Main Entry: **organ**

Pronunciation: 'or-gan

Function: *noun*

Etymology: Middle English, partly from Old English *organa*, from Latin *organum*, from Greek *organon*, literally, tool, instrument; partly from Old French *organe*, from Latin *organum*; akin to Greek *ergon* work -- more at [WORK](#)

Date: before 12th century

1 *a* **archaic** : any of various musical instruments; *especially* : **WIND INSTRUMENT** **b** (1) : a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) : **REED ORGAN** (3) : an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) : any of various similar cruder instruments

2 *a* : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b** : bodily parts performing a function or cooperating in an activity organs>

3 : a subordinate group or organization that performs specialized functions organs of government>

4 : **PERIODICAL**

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Thesaurus Symbol Key

* generally or often considered vulgar

|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	<p>Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart"</p> <p><u>SYNOPSIS:</u> For the first time ever, growth factor inserted into the body grows a new vascular system.</p>	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	<p>Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease"</p> <p><u>SYNOPSIS:</u> A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).</p>	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	<p><u>Circulation</u>, 1998, Editorial, "Angiogenic therapy of the human heart"</p> <p><u>SYNOPSIS</u>: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.</p>	Editorial	Editorial	Editorial
C-4	<p><u>NIH Press Release</u>: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study"</p> <p><u>SYNOPSIS</u>: Researchers at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.</p>	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	<p><u>The Lancet</u>, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb"</p> <p><u>SYNOPSIS</u>: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.</p>	Blood vessels to leg	Balloon Catheter/hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	<p>U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94</p> <p><u>SYNOPSIS:</u> The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.</p>	Formation of new blood vessels	Balloon catheter/hydrogel	Angiogenic growth factors
C-7	<p><u>Harvard University Gazette</u>, 1998, "New Arteries Grown in Diseased Hearts"</p> <p><u>SYNOPSIS:</u> Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.</p>	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: American Heart Association (<http://www.americanheart.org/>)

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's *Circulation: Journal of the American Heart Association*.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients – 14 men and 6 women who were at least 50 years old – who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis – narrowed blood flow due to atherosclerosis – in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein – in a dosage of 0.01 milligrams per kilogram of body weight – was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

Note: This story has been adapted from a news release issued by American Heart Association for journalists and other members of the public. If you wish to quote from any part of this story, please credit American Heart Association as the original source. You may also wish to include the following link in any citation:

<http://www.sciencedaily.com/releases/1998/03/980302070755.htm>

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. (*Circulation*. 1998;97:645-650.)

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.^{1,2} Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

See p 628

In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.³⁻⁹ Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.^{8,10-12}

Gimenez-Gallego et al¹³ succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al¹⁴ isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers¹⁵ successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the

treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.^{4,7}

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E. coli*, a plasmid containing the genetic information being introduced into the microorganisms.¹⁶ These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay⁷ by SDS-PAGE,¹⁶ and the biochemical isolation of FGF-I was confirmed by the Western blot method.¹⁷ Further purification was obtained by HPLC.¹⁸ The factors were lyophilized and stored at -32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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Marban E, Tomaselli GF. allosteric gating: lidocaine unblocks Na channels. *J Clin*

block with mexilitine is and preventing torsade de QT syndrome. *Circulation*.

lidocaine block of LQT-3 103-108.

Bennett PB. Pharmacokinetics. *J Clin Invest*. 1997;

of charge movement in 113-122.

lidocaine on single cardiac 5-874.

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Selected Abbreviations and Acronyms

CHD	= coronary heart disease
EDP	= electronic data processing
FGF	= basic fibroblast growth factor
HPLC	= high-pressure liquid chromatography
IMA	= internal mammary artery
LAD	= left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenic effect of FGF-I.^{19,20} The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,^{4,7} the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 µg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concen-

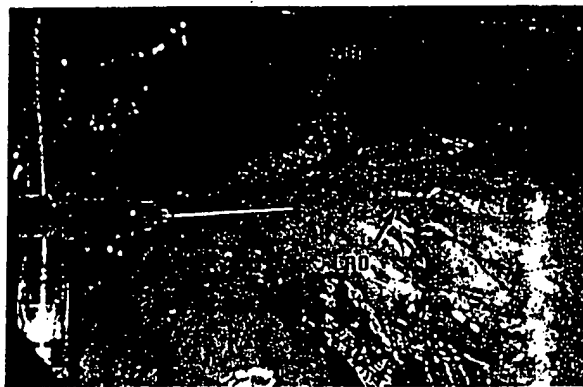


Figure 1. Intraoperative administration of growth factor.

tration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.²¹⁻²³ Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

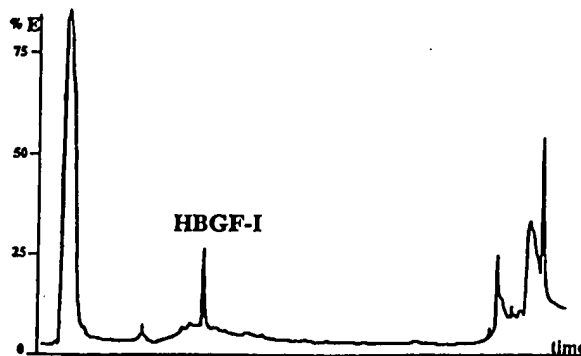


Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

Figure human

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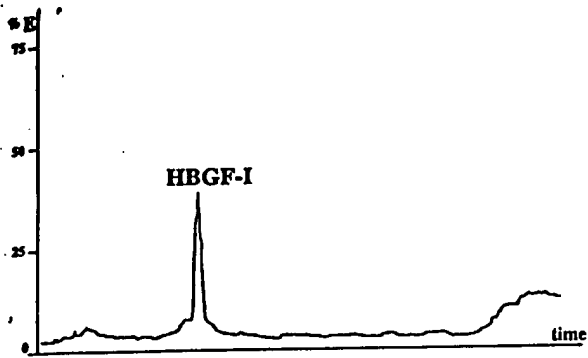


Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

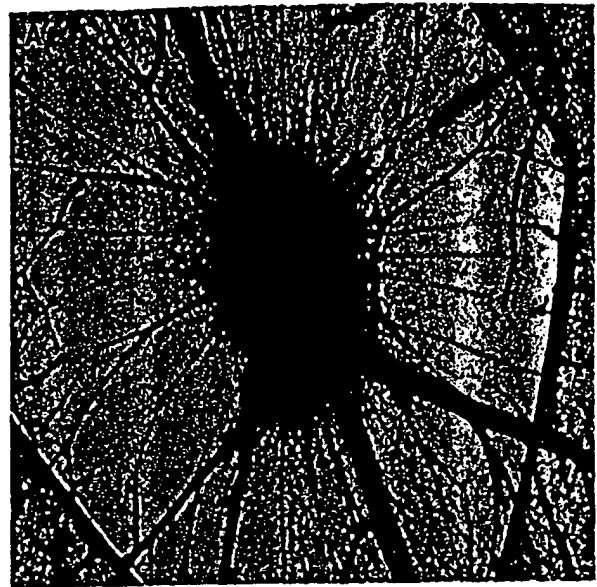
In the chorioallantoic membrane assay, the angiogenic potency of FGF-I could be demonstrated *in vivo*. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group ($n=13$) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

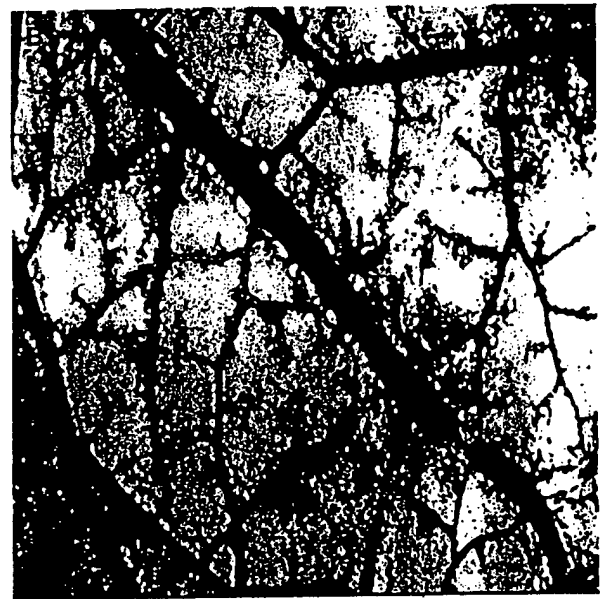
Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I ($n=6$), no signs of induced neoangiogenesis could be found.^{4,7}

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.^{4,7} Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast



10 ng HBGF-I

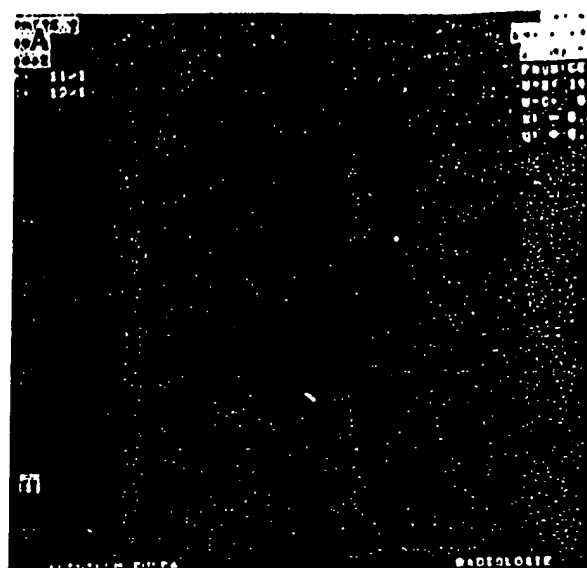


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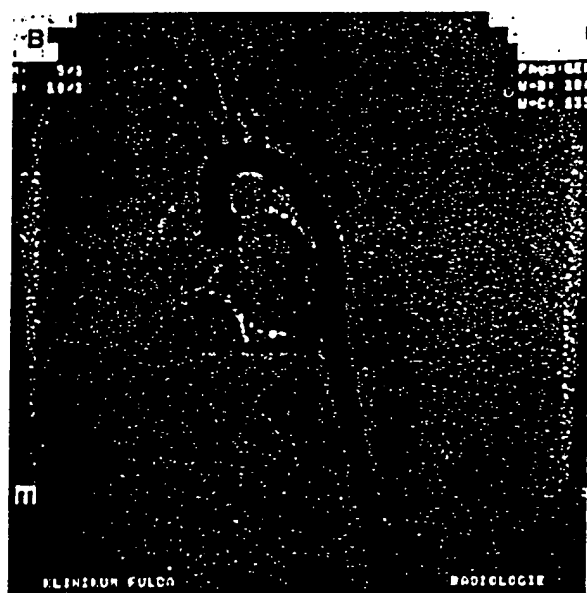
Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ≈ 3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

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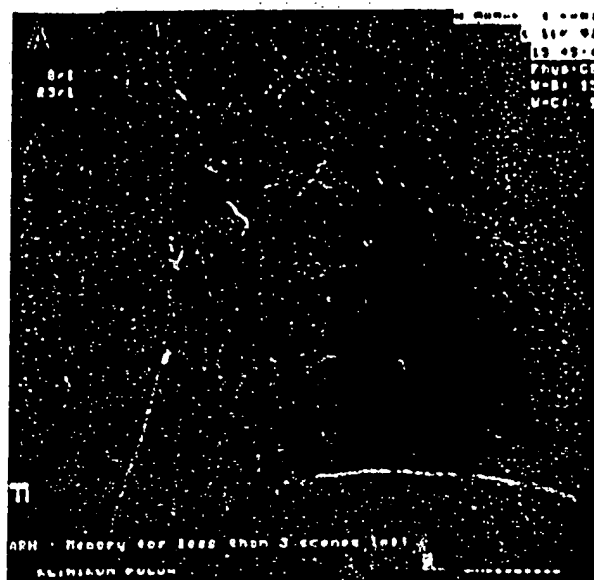


without HBGF-I

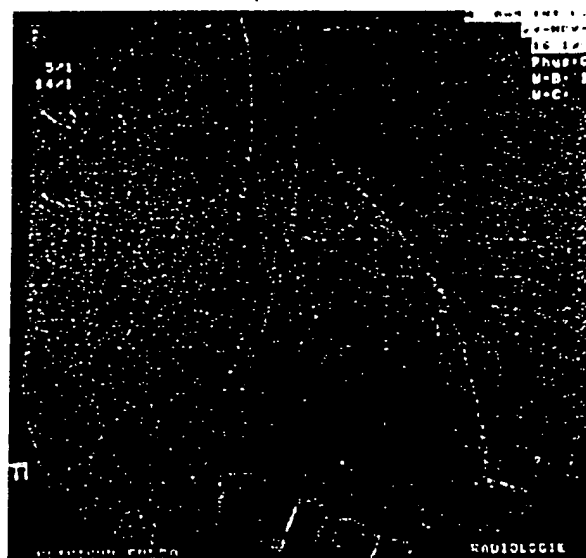
Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about



10 µg/kg HBGF-I



without HBGF-I

Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached

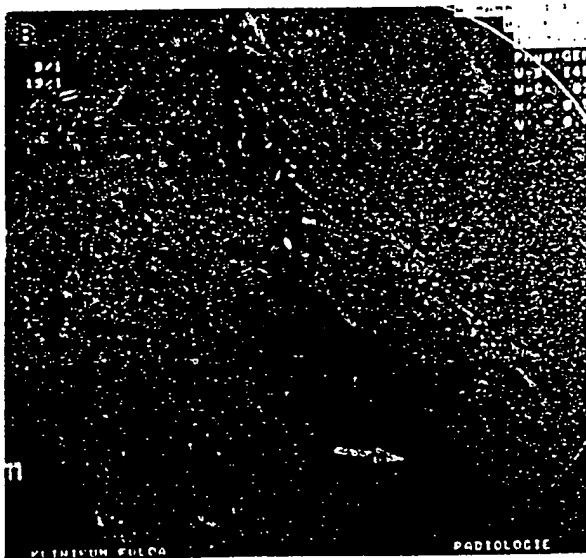
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10 µg/kg HBGF-I



10 µg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological

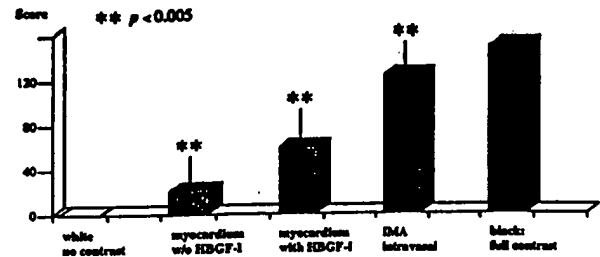


Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.^{8,10-12}

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.^{3,9,27} Yanagisawa-Miwa et al⁹ succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al³ also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al²⁷ produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor-enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,^{4,7} we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,⁴ we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ³H-thymidine into the endothelial cell nuclei using the

method of Klagsbrun and Shing.²⁸ The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.²⁹

On the basis of these *in vitro* and *in vivo* experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis *in situ* in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true *de novo* vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.³⁰ However, before any such possibilities are realized, many more clinical investigations will have to be performed.

References

- Kirklin JW, Subcommittee, and Task Force Members. Guidelines and indications for coronary artery bypass graft surgery: a report of the American College of Cardiology/American Heart Association Task Force on assessment of diagnostic and therapeutic cardiovascular procedures. *J Am Coll Cardiol*. 1991;17:543-589.
- Lytle BW, Loop FD, Cosgrove DM, Easley K, Taylor PC. Long-term (five to twelve years) serial studies of internal mammary artery and saphenous vein coronary bypass graft. *J Thorac Cardiovasc Surg*. 1985;89:248-258.
- Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P. Enhanced angiogenesis and growth of collaterals by *in vivo* administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *J Vasc Surg*. 1992;16:181-191.
- Fasol R, Schumacher B, Schlaudraff K, Hauenstein KH, Seitelberger R. Experimental use of a modified fibrin-glue to induce site-directed angiogenesis from the aorta to the heart. *J Thorac Cardiovasc Surg*. 1994;107:1432-1439.
- Schlaudraff K, Schumacher B, Seitelberger R, v. Specht B-U, Schlosser V, Fasol R. An experimental study of *in situ* and *in vivo* growth of new arteries by the angiogenic growth factor HBGF-1. *Thorac Cardiovasc Surg*. 1992;40:136.
- Schlaudraff K, Schumacher B, Seitelberger R, v. Specht B-U, Schlosser V, Fasol R. Can you grow new arteries for CV surgeons? *J Cardiovasc Surg*. 1992;33:47.
- Schlaudraff K, Schumacher B, v. Specht B-U, Seitelberger R, Schlosser V, Fasol R. Growth of new coronary vascular structures by angiogenic growth factors. *Eur J Cardiothorac Surg*. 1993;7:637-644.
- Thompson JA, Anderson KD, Di Pietro JM, Zwiebel JA, Zametta M, Anderson WF, Maciag T. Site-directed neovessel formation *in vivo*. *Science*. 1988;241:1349-1352.
- Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, Kurashima C, Ito H. Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science*. 1992;257:1401-1402.
- Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M, Schlessinger J. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J*. 1990;9:2685-2692.
- Folkman J, Klagsbrun M. Angiogenic factors. *Science*. 1987;235:442-447.
- Friesel R, Burgess WH, Mehrman T, Maciag T. The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. *J Biol Chem*. 1986;261:7581-7584.
- Gimenez-Gallego G, Rodkey J, Bennett C, Rios-Candelore M, DiSalvo J, Thomas K. Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. *Science*. 1985;230:1385-1388.
- Jaye M, Howk R, Burgess W, Ricca G, Chiu IM, Ravera M, O'Brien S, Modi W, Maciag T, Drohan W. Human endothelial cell growth factor: cloning, nucleotide sequence and chromosome localisation. *Science*. 1986;233:541-545.
- Forough R, Engleka K, Thompson JA, Jackson A, Imamura T, Maciag T. Differential expression in *Escherichia coli* of the alpha and beta forms of heparin-binding acidic fibroblast growth factor 1: potential role of RNA secondary structure. *Biochem Biophys Acta*. 1991;1090:293-298.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680-685.
- Bjerrum OJ, Heegaard NHH, eds. *Handbook of Immunoblotting of Proteins, Volume 1: Technical Descriptions*. Boca Raton, Fla: CRC Press; 1988.
- Gospodarowicz D, Cheng J, Lui GM, Baird A, Böhlent P. Isolation of brain fibroblast growth factor by heparin-sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc Natl Acad Sci U S A*. 1984;81:6963-6967.
- Auerbach R, Kabail L, Knighton D, Folkman J. A simple procedure for the long-term cultivation of chicken embryo. *Dev Biol*. 1974;41:391-394.
- Wilting J, Christ B, Bokeloh M. A modified chorioallantoic membrane (CAM) assay for qualitative and quantitative study of growth factors. *Anat Embryol*. 1991;183:259-271.
- Wilson RF, Johnson MR, Marcus ML, Aylward PE, Skorton DJ, Collins S, White CW. The effect of coronary angioplasty on coronary flow reserve. *Circulation*. 1988;77:873-885.
- Savchenko AP, Pomerantsev EV, Abugov SA, Blank ML. A videodensometric analysis of the characteristics of coronary blood flow using computed digital angiography in patients with ischemic heart disease. *Bull Vsesoiuznogo Kardiolog Nauchn Tsentra AMN SSSR*. 1988;11:96-102.
- Pijls NH, Bos HS, Uijen GJ, Van der Werf T. Is nonionic isotonic iohexol the contrast agent of choice for quantitative myocardial videodensitometry? *Int J Card Imaging*. 1988;3:117-126.
- Chang BL, Drury JK, Meerbaum S, Fishbein MC, Whiting JS, Corday E. Enhanced myocardial washout and retrograde blood delivery with synchronized retroperfusion during acute myocardial ischemia. *J Am Coll Cardiol*. 1987;9:1091-1098.
- Nishimura RA, Rogers PJ, Holmes DR JR, Gehring DG, Bove AA. Assessment of myocardial perfusion by videodensitometry in the canine model. *J Am Coll Cardiol*. 1987;9:891-897.
- Schlaudraff K, von Specht BU, Kolvenbach H, Stegmann T, Schumacher B. Induktion neuer funktioneller Blutgefäße beim Menschen. *Langenbecks Arch Chir Chirurgisches Forum*. 1994;164-172.
- Albes JM, Kletznar T, Kotzerke J, Thiedemann KU, Schäfers HJ, Borst HG. Improvement of tracheal autograft revascularisation by means of fibroblast growth factor. *Ann Thorac Surg*. 1994;57:444-449.
- Klagsbrun M, Shing Y. Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc Natl Acad Sci U S A*. 1985;82:805-809.
- Rosengart TK, Johnson WV, Friesel RE, Clark R, Maciag T. Heparin protects heparin-binding growth factor-1 from proteolytic inactivation *in vitro*. *Biochem Biophys Res Commun*. 1988;152:432-440.
- Ware A, Simons M. Angiogenesis in ischemic heart disease. *Nat Med*. 1997;3:158-164.

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Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.¹ Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.² Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.³⁻⁶ By the mid-1980s, angiogenesis inhibitors began to be discovered.⁷⁻⁹ Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.¹⁰⁻¹²

See p 645

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,^{13,14} (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.¹⁶

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.¹⁷ It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no

evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.¹⁸ Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.^{19,20} Also, FGF can increase expression of (or mobilize) VEGF.²¹

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF²² is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,²³ and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.²⁴ It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

From Children's Hospital, Harvard Medical School, Boston, Mass.

Correspondence to Judah Folkman, MD, Children's Hospital, Harvard Medical School, Hunnewell 103, 300 Longwood Ave, Boston, MA 02115. (Circulation. 1998;97:628-629.)

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References

1. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
2. Gimbrone MA Jr, Cotran RS, Folkman J. Endothelial regeneration and turnover: studies with human endothelial cell cultures. *Ser Haematol*. 1973; 6:453-455.
3. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science*. 1984;223:1296-1298.
4. Maciag T, Mehlman T, Friesel R, Schrieber A. Heparin binds endothelial cell growth factor, the principal mitogen in the bovine brain. *Science*. 1984;225:932-935.
5. Baird A, Bohlen P, Esch F, Baird A, Gospodarowicz D. Acidic fibroblast growth factor (FGF) from bovine brain: amino-terminal sequence and comparison with basic FGF. *EMBO J*. 1985;4:1951-1956.
6. Fets JW, Srydom DJ, Lobb RF, Alderman EM, Bethune JL, Riordan JF, Vallee BL. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry*. 1985;24:5480-5486.
7. Taylor S, Folkman J. Protamine is an inhibitor of angiogenesis. *Nature*. 1982;297:307-312.
8. Crum R, Szabo S, Folkman J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science*. 1985;230: 1375-1378.
9. Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature*. 1990;348:555-557.
10. White CW, Sondheimer HM, Crouch EC, Wilson H, Fan LL. Treatment of pulmonary hemangiomatosis with recombinant interferon alfa-2a. *N Engl J Med*. 1989;320:1197-1200.
11. Folkman J. Successful treatment of an angiogenic disease. *N Engl J Med*. 1989;320:1211-1212.
12. Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med*. 1995;333:1757-1763.
13. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. *N Engl J Med*. 1991; 324:1-8.
14. Horak ER, Leek R, Klenk N, Lejeune S, Smith K, Stuart N, Greenall M, Stepniowska K, Harris AL. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet*. 1992;340:1120-1124.
15. Folkman J. Fighting cancer by attacking its blood supply. *Sci Am*. 1996; 275:150-154.
16. Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Rasvi S, Walsh K, Symes JF. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF₁₂₀ in patient with ischemic limb. *Lancet*. 1996;348:370-374.
17. Schumacher MD, Pocher MD, von Specht BU, Stegmann T. Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation*. 1998;97:645-650.
18. Tuder RM, Flook BE, Voelkel NF. Increased gene expression for VEGF and the VEGF receptors KDR/Fik and Flt in lungs exposed to acute or to chronic hypoxia: modulation of gene expression by nitric oxide. *J Clin Invest*. 1995;95:1798-1809.
19. Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem Biophys Res Commun*. 1992;3: 211-220.
20. Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. *Lab Invest*. 1993;69:508-517.
21. Mandriots SJ, Pepper MS. Vascular endothelial growth factor-induced *in vitro* angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. *J Cell Sci*. 1997;110:2293-2302.
22. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van de Water L, Senger DR. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J Exp Med*. 1991;174:1275-1278.
23. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*. 1996;87:1171-1180.
24. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science*. 1997;277:55-60.

KEY WORDS: Editorials ■ angiogenesis ■ growth substances

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Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bFGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

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of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

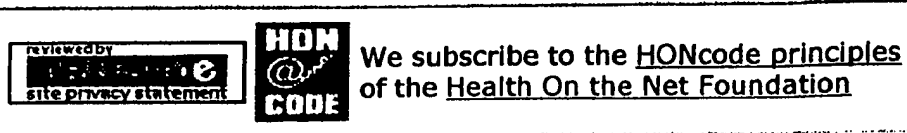
Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study.** November 1, 1999. (Online)
<http://www.nih.gov/news/pr/nov99/nhlbi-01.htm>

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(1 of 1)

United States Patent**5,652,225****Isner****July 29, 1997**

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

Inventors: Isner; Jeffrey M. (Weston, MA)**Assignee:** St. Elizabeth's Medical Center of Boston, Inc. (Boston, MA)**Appl. No.:** 675523**Filed:** July 3, 1996**U.S. Class:**514/44; 604/51; 604/52; 604/53; 536/23.5; 536/23.51;
435/320.1; 435/172.1; 435/172.3; 935/9; 935/22; 935/32;
935/33; 935/34; 935/52; 935/57; 424/93.2**Intern'l Class:**

A01N 047/40

Field of Search:514/44 604/51,52,53 536/23.5,23.51
435/320.1,172.1,172.3,235.1,240.2
935/9,22,32,33,34,52,57 424/93.2

References Cited [Referenced By]

U.S. Patent Documents**EXHIBIT C-5**

<u>5091205</u>	Feb., 1992	Fan	427/2.
<u>5252479</u>	Oct., 1993	Srivastava	435/235.
<u>5304121</u>	Apr., 1994	Sahatjian	604/53.
<u>5328470</u>	Jul., 1994	Nabel et al.	604/101.

Foreign Patent Documents

WO93/00052	Jan., 1993	WO.
WO93/00051	Jan., 1993	WO.

Other References

Fram, et al., Circulation: vol. 86, No. 4, Oct., 1992. Abstract:1515.
Tischer, et al., The Journal of Biological Chemistry; vol. 266, No. 18, Issue of 6/25, pp. 11947-11954, 1991.
Chapman, et al., Circulation Research: vol. 71, No. 1, Jul. 1992, pp. 27-33.
Gal, et al., Circulation: vol. 85, No. 2, Feb. 1992, pp. 756-768.
Nabel, et al., Nature, vol. 362, Apr. 29, 1993, pp. 844-846.
Nabel, et al., Science, Reprint Series Sep. 14, 1990, vol. 249, pp. 1285-1288.
Nabel, et al., Reprint Series Jun. 16, 1989, Science, vol. 244, pp. 1342-1344.
Takeshita, et al., J. Clin. Invest.; vol. 93, Feb. 1994, 662-670.
Riessen, et al., "Human Gene Therapy" 4:749-758 (1993); Published Dec. 1993.
Isner & Rosenfield; Circulation: vol. 88, No. 4, Part 1, Oct. 1993, pp. 1534-1557.
Flugelman, et al., Circulation: vol. 85, No. 3, Mar. 1992, pp. 1110-1117.
Takeshita et al., Circulation: vol. 88, No. 4, Part 2, Oct. 1993; Abstract #1985, p. I-370.
Reissen, et al., Press Release Jul. 14, 1993.
Riessen, et al., European Heart Journal 14:78 (1993), p. 78.
Reissen, et al., Abstract: JACC 21:74A, 1993, American College of Cardiology 42nd Annual Scientific Session; Mar. 14-18, 1993.
Barr & Leiden; TCM 282; Revised Oct. 14, 1993, pp. 57-63.
Steg, et al., Circulation, vol. 90, No. 4, (1994), pp. 1648-1656.

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Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and micodelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., *Lancet* 1:1106-1108, (1989); Wolinsky, et al., *J. Am. Coll. Cardiol.*, 15:475-485 (1990); March et al., *Cardio Intervention*, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., *J. Am. Coll. Cardiol.*, 15:475-481 (1990)).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TGF-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a) is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external iliac artery extends retrograde to its origin from the common iliac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit iliac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit iliac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit iliac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected iliac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit iliac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal iliac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (* $p < 0.05$, ** $p < 0.01$)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSF, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., *Science*, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991) and Folkman, et al., *J. Biol. Chem.* 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., *J. Vasc. Surg.*, 16:181-191 (1992) (bFGF); Pu, et al, *Circulation*, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) (bFGF); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, *Science*, 246:1309-1342 (1989) and Connolly, et al., *J. Biol. Chem.*, 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851-855 (1989) and Conn, et al., *Proc. Natl. Acad. Sci. USA*, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., *Blood*, 81:2767-2773 (1993) and Clauss, et al., *J. Exp. Med.*, 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., *Growth Factors*, 2:9-19 (1989) and Connolly, et al., *J. Clin. Invest.*, 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., *Biochem Biophys Res Commun.*, 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., *J. Clin. Invest.*, 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature* 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. Coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *Bio Techniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R. A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584 (1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

When a hydrophilic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can be protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 $\mu\text{g}/\text{mm}^2$, more preferably between about 0.5 and about 20 $\mu\text{g}/\text{mm}^2$, most preferably between about 1.5 and about 8 $\mu\text{g}/\text{mm}^2$. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm^2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 $\mu\text{g}/\text{mm}^2$ of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 $\mu\text{g}/\text{mm}^2$.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., iliac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore intractable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 μm ; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 μl of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 μl of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 $\mu\text{g}/\mu\text{l}$. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 μl DNA (2 μg DNA/ μl) containing a small amount of ^{35}S -labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction and unincorporated nucleotides were removed by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solveable, TM New England Nuclear, Boston, Mass.) for 3 hours at 50 degree C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 μl of the same lot of labeled DNA (80 μg)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 μl of DNA solution (containing 80 μg of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 μl of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-1.5 μg of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific β -galactosidase (β -gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consists of a full length *Photinus pyralis* luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls β -gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) β -gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from β -gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 μl of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20- μl aliquots prepared from each transfected specimen were mixed in a sample tube with 100 μl of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10 sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: $\text{Luciferase [pg]} = -0.08 + 0.051 \text{ TLU}$. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., *Biotechniques*, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common iliac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external iliac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external iliac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., *J. Clin. Invest.*, 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., *Circ. Res.*, 71:27-33 (1992) and Lim, et al., *Circulation*, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogenesis In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., *J. Clin. Invest.*, 93:662-670 (1994) and Pu, et al., *J. Invest. Surg.*, (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external iliac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external iliac artery extends retrograde to its origin from the common iliac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal iliac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal iliac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., *Science*, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., *Science*, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48×10^6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., *Circulation*, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days post-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., *J. Biol. Chem.* 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., *Proc. Natl. Acad. Sci. USA*, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., *J. Invest. Surg.*, (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal iliac artery of the ischemic limb of 8 animals was transfected with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick layer of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal iliac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal iliac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., *J. Clin. Invest.*, 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal iliac artery. Accordingly, selective internal iliac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., *J. Clin. Invest.*, 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal iliac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., *J. Clin. Invest.*, 93:662-670 (1994). A composite of 5-mm sup. 2 grids was placed over the medial thigh area of the 4-sec angiogram. The total number of grid intersections in the medial thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., *J. Clin. Invest.*, 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 μ m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., *Cardiovasc. Res.*, 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm sup. 2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCl (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using GeneClean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gal), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics.

Results were expressed as means \pm standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of $p < 0.05$ was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48×10^6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 μ g of VEGF protein ($n=3$). In contrast, culture media of .beta.-galactosidase-transfected SMCs ($n=3$) or non-transfected SMCs ($n=3$) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture ($n=3$) and rabbit lilac arteries ($n=3$) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ ($n=3$) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0: 0.17 ± 0.02 vs 0.20 ± 0.06 , $p=ns$). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher than in control group (0.47 ± 0.09 vs 0.34 ± 0.10 , $p < 0.05$) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23 ± 0.12 in VEGF-transfected animals, $p = \text{ns}$). By day 10 post-transfection, the blood pressure ratio for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60 ± 0.12 vs 0.32 ± 0.14 , $p < 0.01$). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70 ± 0.08 vs 0.50 ± 0.18 , $p < 0.05$).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of $233.0 \pm 60.9/\text{mm}^2$ in VEGF-transfected group versus $168.7 \pm 31.5/\text{mm}^2$ in the control group ($p < 0.05$). Analysis of capillary/myocyte ratio disclosed a value of 0.67 ± 0.15 in the VEGF-transfected group versus 0.48 ± 0.10 in the control group ($p < 0.05$).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected iliac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only $< 0.5\%$ of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coli lac Z gene and the SV40 early region nuclear localization sequence (nls). The nls lac Z gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external iliac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming units {pfu} in 2 ml PBS) was transferred to the right iliac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left iliac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used to infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external iliac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral iliac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of β -galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high β -galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10×10^3 ($n=50$ sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote β -galactosidase Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, β -galactosidase gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV. β -gal DNA over endogenous β -galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the β -galactosidase gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV. β -gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the β -galactosidase gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the β -galactosidase gene in 3.10^2 and 3.10^4 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of β -galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10×10^3 to 115.10×10^3 .

Statistics

Results are expressed as mean \pm standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of $p < 0.05$ was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries ($n=15$) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti- α -actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of β -galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti- α -actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear β -galactosidase activity was seen in control arteries.

Morphometric Analysis of nls lacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high β -galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1 \pm 2.3% vs. 0.4 \pm 0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted β -galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10^{sup.3} cells expressed β -galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of β -galactosidase activity in these cases, however, suggested that staining resulted from endogenous β -galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV. β gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express β -galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

College Admission Yield Is Nearly 80%

Women's Studies in Religion Brings New Voices, Perspectives

Bone Drug Lowers Risk of Heart Disease

Virtual Press Room Open for Harvard Conference on Internet & Society

Notes

Labor Economist Myra Strober to Deliver Feminist Economics Lecture at Radcliffe Institute

Police Blotter

A Life of Service

NewsMakers

Peiser Appointed as Professor at Graduate School of Design

Study Finds that Governmental Procedure To Reduce Litigation Actually Leads to More Lawsuits

Knowles Elected Trustee Of Howard Hughes Medical Institute

Faculty To Meet with South Africa's Desmond Tutu, Truth Commission

'Radrugby': Bruised, Battered, Unbowed

Fragments of a Forgotten Past

FAS Administrative, Professional Prizes Honor Staff

New Harvard Features Service Goes Online

Seven Students Win Paine Fellowships

Dental Center's Faculty Practice What They Teach

EXHIBIT C-6

Women In the Ivy League

Conference To Examine the Changing Nature of Journalism

Ann Blair Awarded Radcliffe Junior Faculty Fellowship at Bunting

Exhibit of German Drawings, Watercolors at Sackler Through June 7

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of \$45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of \$45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF₁₆₅ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 µg human plasmid phVEGF₁₆₅ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1998; 348: 370-74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),¹ also known as vascular permeability factor,² and vasculotropin,³ is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.⁴ We have shown⁵ that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF (phVEGF₁₆₅) applied to the hydrogel polymer coating of an angioplasty balloon,⁶ and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to

development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.^{1,7} We now use this strategy in the ischaemic limb of a patient.

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0.26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol⁸ approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

phVEGF₁₆₅ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.⁹ A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of phVEGF₁₆₅-transformed *E coli*, purified with a Qiaeco-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1.75 and 1.85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutaneous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).⁶ A sterile pipette was used to apply 2000 µg plasmid DNA at 10.3 µg/µL in 194.2 µL

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek MD, Robert Schainfeld MD, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh MD, James F Symes MD)
Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

EVIDENCE APPENDIX

ITEM NO. 10

**Supplemental Declaration of Dr. Andrew E. Lorincz
filed November 15, 2004**



PATENT
Appl. No. 09/064,000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia
Serial No.: 09/064,000
Filed: April 21, 1998
For: METHOD AND APPARATUS
FOR INSTALLATION OF
DENTAL IMPLANT

Group Art Unit: 1646

Examiner: Elizabeth Kemmerer, Ph.D.

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail, in an envelope addressed to MAIL STOP AF, Commissioner for Patents, P.O. Box 1450, Arlington, VA 22313-1450 on

NOVEMBER 9, 2004

Gerald K. White 11/9/04
Signature Date

LETTER

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Arlington, VA 22313-1450

Sir:

Enclosed herewith, please find the Supplemental Declaration of Andrew E. Lorincz, M.D.

This Supplemental Declaration is being submitted in an effort to reduce the number of issues in the instant application and thereby expedite the prosecution thereof.

Respectfully submitted,

Date: November 9, 2004

Gerald K. White

Gerald K. White
Reg. No. 26,611

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I Andrew E. Lorincz declare as follows:

1. I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.
2. This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated February 12, 2001, and makes no changes to such previous Declaration.
3. My Curriculum Vitae is attached as Exhibit A to my previous Declaration.
4. In addition to the information set forth in my CV, I provide the following information:

I am familiar with stem cell technology, including bone marrow preparation.

Several publications involving cells are included in my CV; namely, Fluorescent Microscopy of DES-induced Morphologic Transformation in Unfixed, Cultured Cells and Biochemical Genetic Defects.

I performed an unreported study involving assessing stem cell infusion into patients to correct Hurler's Syndrome by transplanting cord blood stem cells. My CV is replete with references to Hurler's Syndrome, as well as other cellular studies.

I am currently Chairman of Vitalflor, a company involved in the observation of cells in the microscopy of vitally stained living organisms, including cells. In this regard, I was granted U.S. Patent Nos. 5,812,314; 6,239,906 B1; and 6,567,214 B2, all of which relate to special stains useful in assessments.

5. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. A copy of such disclosures is attached hereto as Exhibit A.
6. I note that the disclosures referenced in above Paragraph 5 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
7. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit B. Also included in Exhibit B is a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary, a service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe

that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.

8. I have read and understood the claims set forth in Exhibit C and have been informed that such claims are present in the above-referenced patent application. It is my opinion that those skilled in the medical arts, reading such claims would understand that cells including stem cells, are species of living organisms.
9. The publication in attached Exhibit D illustrates that placement of a growth factor, including cells, and more specifically, stem cells, in a human patient forms soft tissue, such as an artery. This publication reports work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.
10. Based upon above Paragraphs 5-9, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery.
11. Based upon above Paragraphs 5-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.
12. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 11-8-04

Andrew E. Lorincz
Andrew E. Lorincz

**SUPPLEMENTAL
EXHIBIT A**

DISCLOSURES

**APPLICATION
SERIAL NO. 09/064,000**

SUPPLEMENTAL EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylolation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

SUPPLEMENTAL EXHIBIT B

DEFINITIONS

SUPPLEMENTAL EXHIBIT B

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

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by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Growth factor: a substance (as a vitamin B₁₂ or an interleukin)
that promotes growth and especially cellular growth

**SUPPLEMENTAL
EXHIBIT C**

CLAIMS

EXHIBIT C

CLAIMS **APPLICATION SERIAL NO. 09/064,000**

382. A method for producing a desired soft tissue in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud in said body of said human patient; and
 - (c) Growing said desired soft tissue from said bud.
383. The method of claim 382, wherein said cells are multifactorial and non-specific.
384. The method of claim 383, wherein said cells comprise stem cells.
385. The method of claim 382 further comprising forming a new artery.
386. The method of claim 383 further comprising forming a new artery.
387. The method of claim 382, wherein said soft tissue comprises mesodermal tissue.
388. The method of claim 382, wherein said soft tissue comprises an artery.

**SUPPLEMENTAL
EXHIBIT D**

PUBLICATIONS

Clinical Investigation and Reports

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow-derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow-derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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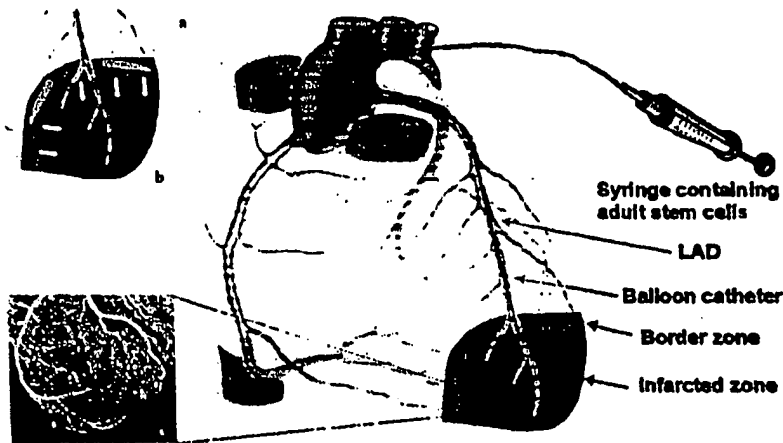


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. *a*, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. *b*, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. *c*, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery ($n=4$), left circumflex coronary artery ($n=3$), or right coronary artery ($n=13$). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty ($n=20$) and subsequent stent implantation ($n=19$). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients ($n=10$). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7 ; this consisted of $0.65 \pm 0.4\%$ AC133-positive cells and $2.1 \pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ³)	2.8±2.2

Values are mean±SD or number of patients.

NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index P_{170}/ESV was calculated by dividing LV systolic pressure (P_{170}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility Indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11-14,18,20-23} Moreover, transendothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24-26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells (≤1%), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

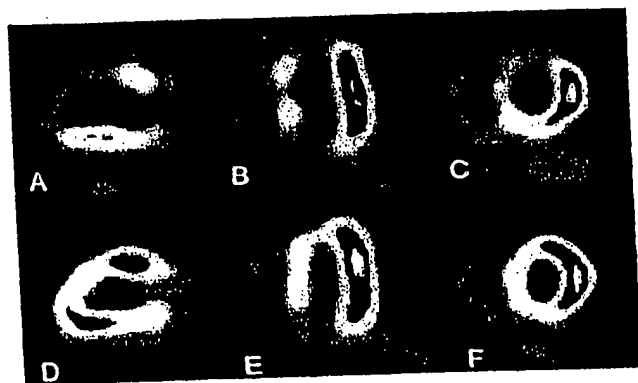


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ²⁰¹thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m ²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility Indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P _{max} /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
²⁰¹ Thallium scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells¹⁻¹²; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of *in vitro* amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility ($P_{1/2}/ESV$ and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

References

- Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction: experimental observations and clinical implications. *Circulation*. 1990;81:1161-1172.
- Ertl G, Gaudron P, Hu K. Ventricular remodeling after myocardial infarction: experimental and clinical studies. *Basic Res Cardiol*. 1993;88:125-137.
- Quinini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. *N Engl J Med*. 2002;346:5-15.
- Leor J, Patterson M, Quinones MJ, et al. Transplantation of fetal myocardial tissue into infarcted myocardium of rat: a potential method for repair of infarcted myocardium? *Circulation*. 1996;94(suppl II):332-336.
- Murry CE, Wiseman RW, Schwartz SM, et al. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest*. 1996;98:2512-2523.
- Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med*. 1998;4:929-933.
- Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation*. 1999;100(suppl II):247-256.
- Blau HM, Brazzelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? *Cell*. 2001;105:829-841.
- Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;103:369-377.
- Goodell MA, Jackson KA, Majka SM, et al. Stem cell plasticity in muscle and bone marrow. *Ann NY Acad Sci*. 2001;938:208-218.
- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-705.
- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA*. 2001;98:10344-10349.
- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430-436.
- Tomita S, Mickle DA, Weisel RD, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg*. 2002;123:1132-1135.
- Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. *Lancet*. 2001;357:279-280.
- Hamano K, Nishida M, Hirata K, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J*. 2001;65:845-847.
- Strauer BE, Brehm M, Zeus T, et al. Myocardial regeneration after intracoronary transplantation of human autologous stem cells following acute myocardial infarction. *Disch med Wschr*. 2001;126:932-938.
- Wang JS, Shum-Tim D, Chedrawy E, et al. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg*. 2001;122:699-705.
- Sheehan FH, Bolson EL, Dodge HT, et al. Advantages and applications of the Centerline method for characterizing regional ventricular function. *Circulation*. 1986;74:293-305.
- Sussman M. Cardiovascular biology: hearts and bones. *Nature*. 2001;410:640-641.
- Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-98.
- Kamihata H, Matsubara H, Nishiue T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046-1052.
- Ferrari G, Cusella-De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*. 1998;279:1528-1530.
- Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634-637.
- Robinson SW, Cho PW, Levisky HI, et al. Arterial delivery of genetically labelled skeletal myoblasts to the murine heart: long-term survival and phenotypic modification of implanted myoblasts. *Cell Transplant*. 1996;5:77-91.
- Bitner RE, Schofer C, Weipoltshammer K, et al. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat Embryol (Berl)*. 1999;199:391-396.
- Jiang Y, Jahagirdar B, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;20:1-12.
- Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood*. 2001;98:2615-2625.
- Condorelli G, Borello U, De Angelis L, et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. *Proc Natl Acad Sci USA*. 2001;98:10733-10738.
- Szallasi ZJ, Bass MJ, Van Zant G, et al. Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood*. 1999;93:1557-1566.
- Gregg DE, Fisher LC. Blood supply to the heart. In: *Handbook of Physiology*. Vol 2. Washington, DC: American Physiological Society; 1963:chap 44, 1517-1584.
- Strauer BE. Myocardial oxygen consumption in chronic heart disease: role of wall stress, hypertrophy and coronary reserve. *Am J Cardiol*. 1979;44:730-740.
- Toma C, Pittenger MF, Byrne BJ, et al. Adult human mesenchymal stem cells differentiate to a striated muscle phenotype following arterial delivery to the murine heart. *Circulation*. 2000;102(suppl II):II-683. Abstract.
- Hendriks PJ, Martens CM, Hagenbeek A, et al. Homing of fluorescently labeled murine hematopoietic stem cells. *Exp Hematol*. 1996;24:129-140.
- Ren G, Michael LH, Entman ML, et al. Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem*. 2002;50:71-79.
- Li RK, Mickle DA, Weisel RD, et al. Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. *Ann Thorac Surg*. 2001;72:1957-1963.
- Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002;53:31-47.
- Allgöwer M. *The Cellular Basis of Wound Repair*. Springfield, Ill: Charles C. Thomas; 1956.
- Xie Y, Zhou T, Shen W, et al. Soluble cell adhesion molecules in patients with acute coronary syndrome. *Chin Med J*. 2000;113:286-288.
- Soeki T, Tamura Y, Shinohara H, et al. Serial changes in serum VEGF and HGF in patients with acute myocardial infarction. *Cardiology*. 2000;93:168-174.
- Sheiban I, Frangasso G, Rosano GMC, et al. Time course and determinants of left ventricular function recovery after primary angioplasty in patients with acute myocardial infarction. *J Am Coll Cardiol*. 2001;38:464-471.

EVIDENCE APPENDIX

ITEM NO. 11

**Second Supplemental Declaration of Dr. Andrew E. Lorincz
cited by Appellant as Exhibit D in the Response filed June 26, 2006**



APPL. SERIAL NO. 09/064,000
SECOND SUPPL LORINCZ DECLARATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I Andrew E. Lorincz declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001. Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my

previous Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Second Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Second Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 5 June 2006

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL’s), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT C

CLAIMS

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 382 A method for producing and integrating tissue consisting of a desired soft tissue at a selected site in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud at said selected site in said body of said human patient; and
 - (c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
- Claim 383 The method of claim 382, wherein said cells are multifactorial and non-specific.
- Claim 384 The method of claim 383, wherein said cells comprise stem cells.
- Claim 385 The method of claim 382 further comprising forming a new artery.
- Claim 386 The method of claim 383 further comprising forming a new artery.
- Claim 387 The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

- Claim 388 The method of claim 382, wherein said soft tissue comprises an artery.
- Claim 389 The method of claim 382, wherein said cells comprise stem cells.
- Claim 390 The method of claim 389, wherein said soft tissue comprises an artery.
- Claim 391 The method of claim 382, wherein said cells comprise pluripotent cells.
- Claim 392 The method of claim 391, wherein said soft tissue comprises an artery.
- Claim 393 The method of claim 391, wherein said cells comprise stem cells.
- Claim 394 The method of claim 393, wherein said stem cells are multifactorial and non-specific.
- Claim 395 The method of claim 382, wherein said cells are injected into said body.
- Claim 396 The method of claim 382, wherein said cells are locally placed into said body.
- Claim 397 The method of claim 396, wherein said cells comprise stem cells.
- Claim 398 The method of claim 396, wherein said cells are injected intramuscularly.
- Claim 399 The method of claim 397, wherein said stem cells are injected intramuscularly.

- Claim 400 The method of claim 388 further comprising determining blood flow through said new artery.
- Claim 401 The method of claim 388 further comprising observing said new artery.
- Claim 402 The method of claim 399, wherein said selected site comprises a leg of said patient.

EVIDENCE APPENDIX

ITEM NO. 12

**Third Supplemental Declaration of Dr. Andrew E. Lorincz
cited by Appellant as Exhibit B in the Response filed April 30, 2007**



A.L. SERIAL NO. 09/064,000
THIRD SUPPL LORINCZ DECLARATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**THIRD SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I Andrew E. Lorincz declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001. Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my

previous Declaration. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Third Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Third Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit C without need for resorting to undue experimentation.
8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2-22-07

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF- β), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which

promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have

grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 47, LINE 22 – PAGE 48, LINE 15

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell can not [sic] be obtained, the damaged cell can be repaired by excision, alkylation, transition or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of morphogenesis. The foregoing can be repeated without the patient's own

cells if universal donor cells such a [sic] germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foregoing procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some case, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/064,000

PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term “cell nutrient culture” as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF₁₆₅, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo

Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT C

CLAIMS

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 382 A method for producing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient comprising:
- (a) Placing cells in said body of said human patient;
 - (b) Forming a bud at said selected site in said body of said human patient; and
 - (c) Growing said desired soft tissue which integrates itself into said body of said human patient from said bud.
- Claim 383 The method of claim 382, wherein said cells are multifactorial and non-specific.
- Claim 384 The method of claim 383, wherein said cells comprise stem cells.
- Claim 385 The method of claim 382 further comprising forming a new artery.
- Claim 386 The method of claim 383 further comprising forming a new artery.
- Claim 387 The method of claim 382, wherein said soft tissue comprises mesodermal tissue.

- Claim 388 The method of claim 382, wherein said soft tissue comprises an artery.
- Claim 389 The method of claim 382, wherein said cells comprise stem cells.
- Claim 390 The method of claim 389, wherein said soft tissue comprises an artery.
- Claim 391 The method of claim 382, wherein said cells comprise pluripotent cells.
- Claim 392 The method of claim 391, wherein said soft tissue comprises an artery.
- Claim 393 The method of claim 391, wherein said cells comprise stem cells.
- Claim 394 The method of claim 393, wherein said stem cells are multifactorial and non-specific.
- Claim 395 The method of claim 382, wherein said cells are injected into said body.
- Claim 396 The method of claim 382, wherein said cells are locally placed into said body.
- Claim 397 The method of claim 396, wherein said cells comprise stem cells.
- Claim 398 The method of claim 396, wherein said cells are injected intramuscularly.
- Claim 399 The method of claim 397, wherein said stem cells are injected intramuscularly.

- Claim 400 The method of claim 388 further comprising determining blood flow through said new artery.
- Claim 401 The method of claim 388 further comprising observing said new artery.
- Claim 402 The method of claim 399, wherein said selected site comprises a leg of said patient.
- Claim 403 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:
- (a) locally injecting stem cells into said body at said selected site;
 - (b) forming a bud at said selected site; and
 - (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.
- Claim 404 The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
- Claim 405 The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.

Claim 406

The method of claim 402, wherein said desired soft tissue comprises an artery

EVIDENCE APPENDIX

ITEM NO. 13

**Fourth Supplemental Declaration of Dr. Andrew E. Lorincz cited by
Appellant as Exhibit E in the Response filed November 28, 2007**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 09/064,000)	
)	Examiner: Elizabeth C. Kemmerer, Ph.D.
Filed: April 21, 1998)	
)	
For: METHOD FOR GROWTH)	
OF SOFT TISSUE)	

**FOURTH SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I Andrew E. Lorincz declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. My Curriculum Vitae is attached as Exhibit A to my Declaration of February 12, 2001. Paragraph 4 of my Declaration and my Supplemental Declaration of November 8, 2004 provide additional information regarding my background and experience.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; at page 37, lines 19-25; at page 44, line 19 through page 46, line 16; and at page 47, line 22 through page 48, line 15. Such disclosures are the same as I read and understood in my

previous Declaration. A copy of such disclosures was attached to my Third Supplemental Declaration as Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures was attached to my Third Supplemental Declaration as Exhibit B.

4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue, and more specifically, to a method of using a cell, such as a stem cell, to grow soft tissue, such as an artery.
5. I have read and understood the claims set forth in the attached Fourth Supplemental Declaration Exhibit A and have been informed that such claims will be concurrently presented in the above-referenced patent application with this Fourth Supplemental Declaration.
6. Based upon above Paragraphs 3-5, it is my opinion that introducing a growth factor, including cells, and more specifically, stem cells, in the body of a human patient will predictably result in the growth of soft tissue, such as an artery, which will integrate itself into pre-existing tissue of the body thereby forming a unified whole.

7. Based upon above Paragraphs 3-5, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit A without need for resorting to undue experimentation.
8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 11-13-07

Andrew E. Lorincz
Andrew E. Lorincz, M.D.

FOURTH SUPPLEMENTAL DECLARATION

EXHIBIT A

CLAIMS

EXHIBIT A
CLAIMS
APPLICATION SERIAL NO. 09/064,000

- Claim 403 A method for growing and integrating tissue consisting of desired soft tissue at a selected site in a body of a human patient wherein said desired soft tissue comprises a desired artery comprising the steps of:
- (a) locally injecting stem cells into said body at said selected site;
 - (b) forming a bud at said selected site; and
 - (c) growing said desired artery from said bud wherein said artery integrates itself into said body of said human patient at said selected site.
- Claim 404 The method of claim 403, wherein said selected site comprises a damaged site in a leg of said patient and said stem cells are injected intramuscularly.
- Claim 405 The method of claim 403, wherein said selected site comprises a damaged site in a heart of said patient and said stem cells are injected intramuscularly.
- Claim 407 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from bone marrow.

- Claim 408 The method of claim 407, wherein said bone marrow is from said patient.
- Claim 409 The method of claim 403, wherein said stem cell comprises a living stem cell harvested from blood.
- Claim 410 The method of claim 409, wherein said blood is from said patient.
- Claim 411 The method of claim 403 further comprising determining blood flow through said desired artery.
- Claim 412 The method of claim 403 further comprising observing said desired artery.

EVIDENCE APPENDIX

ITEM NO. 14

**Second Supplemental Declaration of Dr. Andrew E. Lorincz
(originally filed in co-pending application Serial No. 10/179,589) and
cited by Appellant as Exhibit A in the Letter filed May 25, 2007**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)	
Serial No.: 10/179,589)	Group Art Unit: 1646
Filed: June 25, 2002)	Examiner: Elizabeth Kemmerer
For: METHOD FOR GROWING)	
HUMAN ORGANS AND)	
SUBORGANS)	

**SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. My Curriculum Vitae is attached was Exhibit A to my Declaration of November 8, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of June 5, 2006 provide additional information regarding my background and experience.
3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Second Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Second Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Second Supplemental Declaration Exhibit C.
6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Second Supplemental Declaration Exhibit E. Note that in two of the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Second Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 4-19-07

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

SECOND SUPPLEMENTAL
DECLARATION

EXHIBIT A

MARCH 7, 2007

OFFICE ACTION

Paragraph 11, pages 7-9

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6 , and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage as purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed to the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

Application/Control Number: 10/179,589

Page 9

Art Unit: 1647

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

SECOND SUPPLEMENTAL DECLARATION

EXPERIENCE

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylolation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

SECOND SUPPLEMENTAL
DECLARATION

EXHIBIT C

ADDITIONAL
DISCLOSURES

EXHIBIT C
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1- 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEGF or BMP growth factors produced by VEGF or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 73 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8-17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

SECOND SUPPLEMENTAL
DECLARATION
EXHIBIT D

COMMISSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6 , respectively.

SECOND SUPPLEMENTAL
DECLARATION

EXHIBIT F

PUBLICATIONS (3)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemia series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under

therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu\text{g.} \times 10^{-7}$ per cell

	DNAP 18 obs. on 18 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8.54	6.33	0.75
S.E. of obs.	2.89	3.03	0.326
Observed range	4.0-15.0	2.1-13.5	0.43-1.9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8.75	7.59	0.80
S.E. of obs.	3.05	3.72	0.30
Observed range	3.9-17.4	2.6-17.4	0.3-1.8

Table 2. Cases of pernicious anaemia and other megaloblastic anaemias

NAP in $\mu\text{g.} \times 10^{-7}$ per cell

Group as a whole	Mean S.E. Observed range	DNAP 28 obs. on 12 cases	RNAP 28 obs. on 13 cases	Ratio DNAP/RNAP 28 obs. on 13 cases
		12.6 4.58 6.6-22.8		
Group prior to therapy	Mean	12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
	S.E.	12.57	13.38	1.06
	Observed range	4.17 8.1-22.8	5.19 7.5-25.1	0.249 0.69-1.5
Group during the course of therapy	Mean	17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
	S.E.	12.63	9.09	0.73
	Observed range	4.36 6.6-18.8	4.21 2.3-17.6	0.198 0.35-1.0

Table 3. t test of significance between means

	P	DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	Degrees of freedom	44	44	46
Megaloblastic series before therapy compared with normal	P	<0.001	<0.001	0.2-0.1
Megaloblastic series during therapy compared with normal	Degrees of freedom	28	29	30
Megaloblastic series before and during therapy compared	P	0.01-0.001	0.05-0.02	0.8-0.7
	Degrees of freedom	33	33	34
	P	0.7-0.6	0.05-0.02	<0.001
	Degrees of freedom	27	24	26

REFERENCES

- Davidson, J. N., Leslie, I. & White, J. C. (1947). *Biochem. J.* 41, xxvi.
 Davidson, J. N., Leslie, I. & White, J. C. (1948). *J. Path. Bact.* 50, 1.
 Mirsky, A. E. & Ris, H. (1949). *Nature, Lond.*, 163, 666.
 Vendrely, R. & Vendrely, C. (1948). *Experientia*, 4, 434.
 Vendrely, R. & Vendrely, C. (1949). *Experientia*, 5, 327.

Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (Department of Biochemistry, University of Oxford)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. Rat heart aconitase

Time (min.)	Citric acid (μmol.)	
	0	60
Additions:		
cis-Aconitate (5 μmol.)	0.21	3.90
cis-Aconitate + 'active' fraction	0.08	3.96
Citrate (5 μmol.)	4.90	4.34
Citrate + 'active' fraction	5.27	4.38

Table 2. Pig heart isocitric dehydrogenase

	<i>E₅₀₀</i> mμ. (max. value)
DL-isocitrate only	0.076-0.065
Same + 'active' fraction	0.075
Same + p-chloromercuribenzoic acid 1.33 × 10 ⁻³ M	0.004

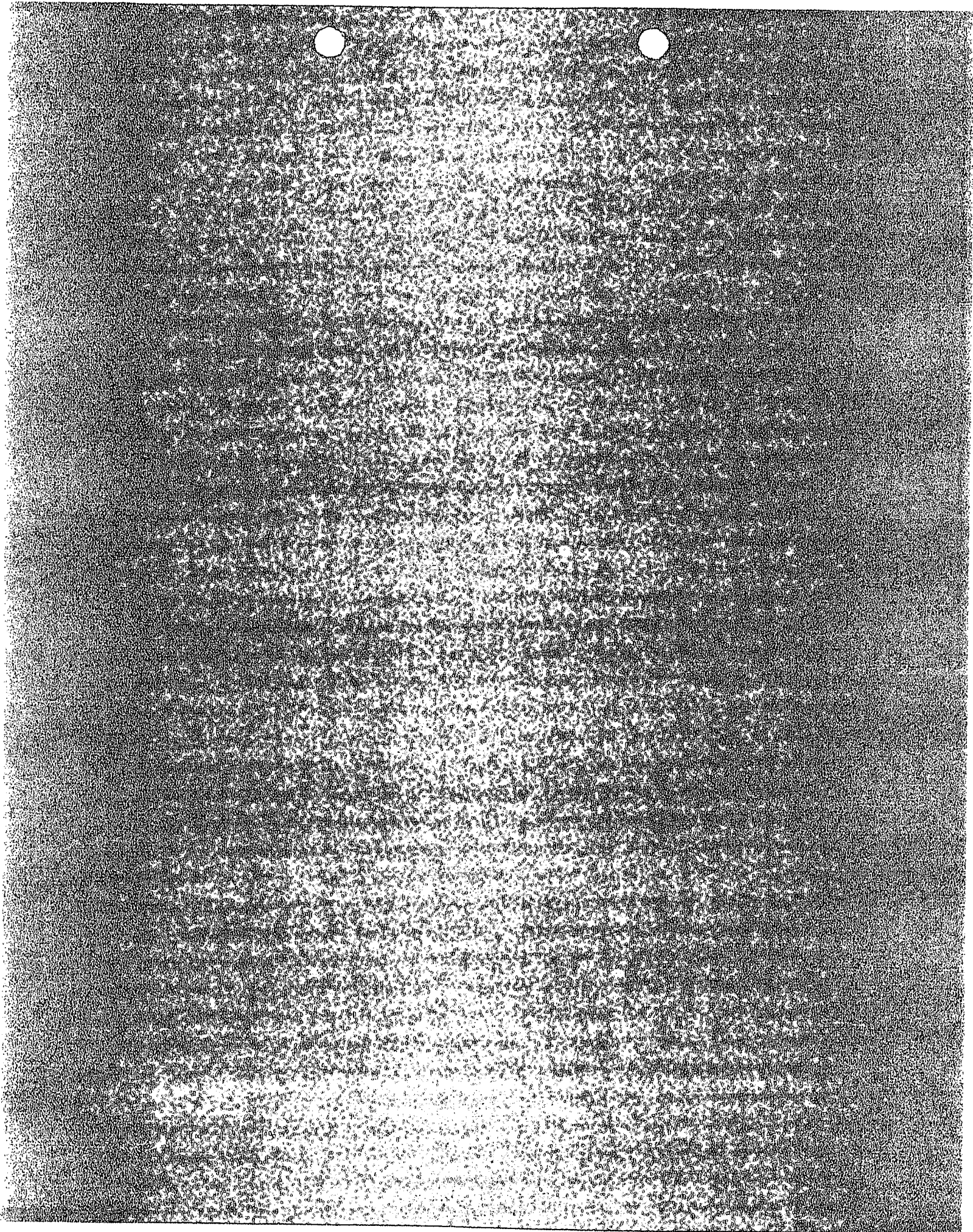
Table 3. Pig heart oxalosuccinic decarboxylase
(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min.
at 13.5°C. Net values)

	CO ₂ (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

REFERENCES

- Adler, E., v. Euler, H., Günther, G. & Plass, M. (1939). *Biochem. J.* 33, 1028.
 Buffa, P. & Peters, R. A. (1949). *J. Physiol.* 110, 488.
 Buffa, P., Peters, R. A. & Wakelin, R. W. (1950). In course of publication.
 Johnson, W. A. (1939). *Biochem. J.* 33, 1046.
 Liébecq, C. & Peters, R. A. (1949). *Biochim. Biophys. Acta*, 3, 215.
 Martius, O. (1949). *Liebigs Ann.* 561, 227.
 Ochoa, S. & Weiss-Tabori, E. (1948). *J. biol. Chem.* 174, 123.



Appendix

Nucleic Acids

Nucleic Acids

Content and Distribution

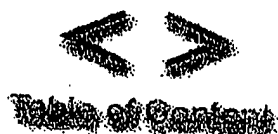
Nucleic acids in an average human cell

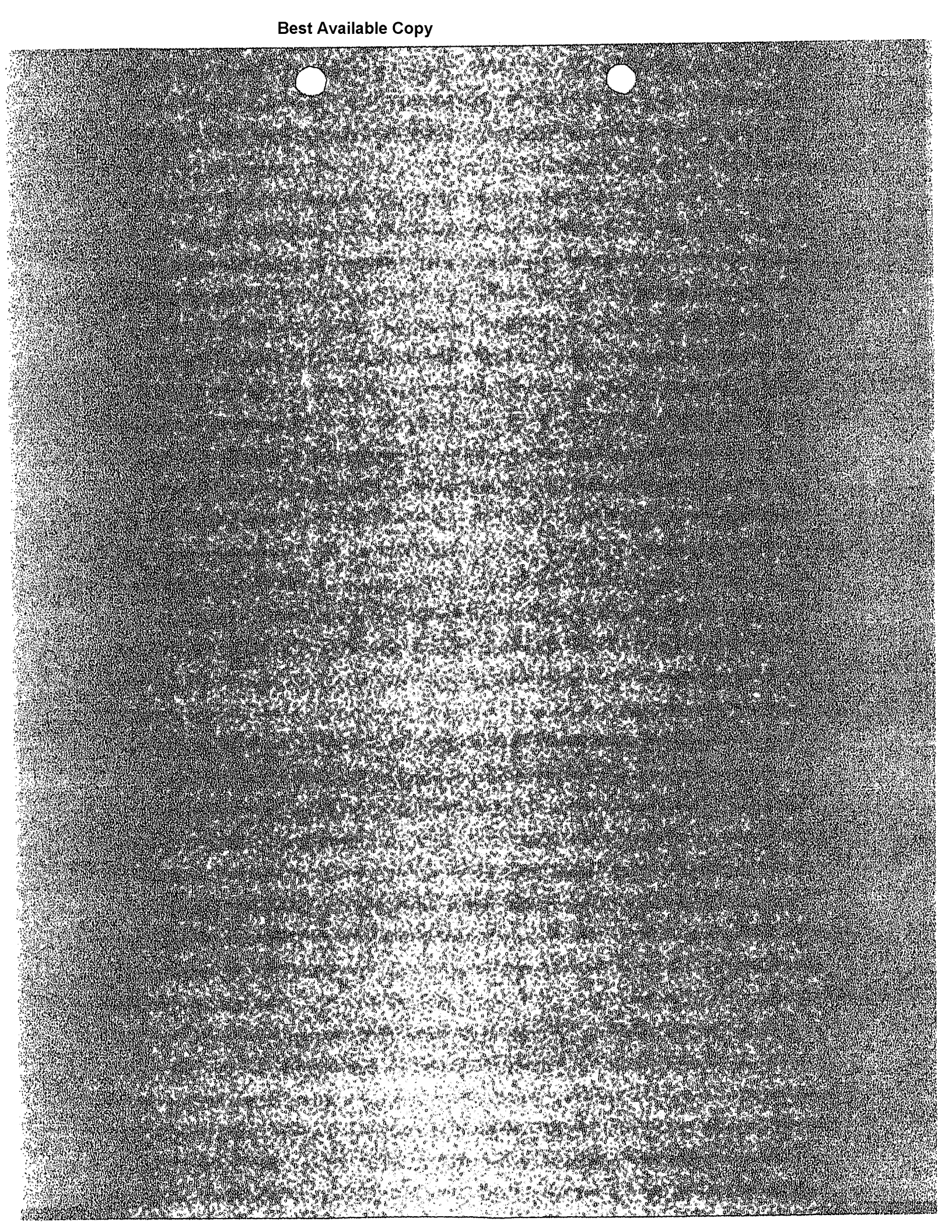
DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	$0.51.0 \times 10^5$
	1.5×10^4
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
Ratio of DNA:RNA in nucleus	~14% of total RNA
Number of mRNA moleculesc	~ 2:1
Number of different mRNA species	$0.2 \ 1.0 \times 10^6$
Low abundance mRNA (5 15 copies/cell)	$1.0 \ 3.4 \times 10^4$
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
Abundance of each message for:	<10 different messages
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

- a 30 – 60 µg/ml blood for human leukocytes.
b 1 – 5 µg/ml blood for human leukocytes.
c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells))	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6





UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1 µg	1 nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA $\sim 3 \times 10^6$ nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (diploid)	0.03 pg
<i>E. coli</i>	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
<i>S. cerevisiae</i>	5 μm	66 μm^3
<i>S. pombe</i>	2 x 7 μm	22 μm^3
Mammalian cell	10-20 μm	500-4,000 μm^3
<i>E. coli</i>	1 x 3 μm	2 μm^3
Mammalian mitochondrion	1 μm	0.5 μm^3
Mammalian nucleus	5-10 μm	66-500 μm^3
Plant chloroplast	1 x 4 μm	3 μm^3
Bacteriophage lambda	50 nm (head only)	6.6 x 10 ⁻⁵ μm^3
Ribosome	30 nm diameter	1.4 x 10 ⁻⁵ μm^3
Globular monomeric protein	5 nm diameter	6.6 x 10 ⁻⁸ μm^3

III. Some useful concentrations

Total cell protein concentration Detergent soluble protein = 1-2 mg/ 10⁷ mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

Abundant transcription factor

Rare transcription factor

1 nM (100,000 copies/ nucleus)

10 pM (1,000 copies/ nucleus)

Serum

50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	μg
10,000 Da protein	1

100,000 Da protein

10

Protein/ DNA conversions1 kb of DNA encodes 333 amino acids $\approx 3.7 \times 10^4$ Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10 -30 pg
Proportion of total RNA in nucleus	$\sim 14\%$
DNA:RNA in nucleus	$\sim 2:1$
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5 - 1×10^5
Active genes	1.5×10^4
mRNA molecules	2×10^5 - 1×10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (μ g)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)			
	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocytes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O ₂ & CO ₂ transport
Cells per ml	4-7 x 10 ⁶	3-4 x 10 ⁸	5 x 10 ⁹
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNA content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 10⁶ per ml in cases of immunosuppression, to 40 x 10⁶ during inflammation, to 500 x 10⁶ during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

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Mit Urteil vom 12. Mai 1998 hat das Landgericht Hamburg entschieden, dass man durch die Ausbringung eines Links die Inhalte der gelinkten Seite ggf. mit zu verantworten hat. Dies kann - so das LG - nur dadurch verhindert werden, dass man sich ausdrücklich von diesen Inhalten distanzliert. Wir haben auf verschiedenen Seiten dieser Homepage Links zu anderen Seiten im Internet gelegt. Für all diese Links gilt: "Wir möchten ausdrücklich betonen, dass wir keinerlei Einfluss auf die Gestaltung und die Inhalte der gelinkten Seiten haben. Deshalb distanzieren wir uns hiermit ausdrücklich von allen Inhalten der gelinkten Seiten auf der Website inklusive aller Unterseiten und machen uns ihre Inhalte nicht zu eigen." Diese Erklärung gilt für alle auf der Homepage ausgebrachten Links und für alle Inhalte der Seiten, zu denen Links führen.

EVIDENCE APPENDIX

ITEM NO. 15

**Office Action issued on February 22, 2006 in co-pending application
Serial No. 09/794,456 by Examiner Kemmerer, page 6, lines 1-8,
cited by Appellant as Exhibit B in Appeal Brief filed herewith**

Art Unit: 1646

products, not method steps. The issue here is not whether or not workers in this technology already knew the features of the cells recited in the claims; rather, the issue is that the instant specification did not set forth contemplation of a method step wherein cells were administered intravenously, intraluminally, or via angioplasty. As discussed in the previous paragraph, the instant specification did not set forth contemplation of such method steps. The claims are being examined to the extent they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. Furthermore,

MPEP § 2163.02 reads:

"An Applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. See Lockwood v. American Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention."

In the instant case, none of these criteria have been met. There was no reduction to practice, and the specification only refers to method steps involving proteins, genes and "genetic material," *but not cells*, as being useful in intravenous, intraluminal and angioplasty delivery. Therefore, the rejection is maintained.

35 U.S.C. § 112, First Paragraph – Enablement

EVIDENCE APPENDIX

ITEM NO. 16

**Nabel U.S. Patent No. 5,328,470
cited by Appellant as Reference AD in the Information
Disclosure Statement filed February 15, 2001**



US005328470A

United States Patent [19][11] **Patent Number:** 5,328,470

Nabel et al.

[45] **Date of Patent:** Jul. 12, 1994**[54] TREATMENT OF DISEASES BY
SITE-SPECIFIC INSTILLATION OF CELLS
OR SITE-SPECIFIC TRANSFORMATION OF
CELLS AND KITS THEREFOR****[75] Inventors:** Elizabeth G. Nabel; Gary J. Nabel,
both of Ann Arbor, Mich.**[73] Assignee:** The Regents of the University of
Michigan, Ann Arbor, Mich.**[21] Appl. No.:** 741,244**[22] Filed:** Jul. 26, 1991**Related U.S. Application Data****[63]** Continuation-in-part of Ser. No. 724,509, Jun. 28, 1991,
which is a continuation-in-part of Ser. No. 331,336,
Mar. 31, 1989, abandoned.**[51] Int. Cl.³** A61M 29/00**[52] U.S. Cl.** 604/101; 604/96;
606/194**[58] Field of Search** 604/52, 53, 96, 97,
604/101, 181, 269, 280; 424/424, 425, 93 B;
514/120; 606/192, 174; 128/656, 658**[56] References Cited****U.S. PATENT DOCUMENTS**

4,182,330	1/1980	Michaels	424/473
4,332,893	6/1982	Rosenberg .	
4,351,337	9/1982	Sidman	424/425
4,353,888	10/1982	Sefton .	
4,636,195	1/1987	Wolinsky .	
4,824,436	4/1989	Wolinsky .	
5,049,132	9/1991	Shaffer et al.	604/101

FOREIGN PATENT DOCUMENTS

0273085	7/1988	European Pat. Off. .	
9002580	3/1990	World Int. Prop. O.	604/52

OTHER PUBLICATIONS

Science, vol. 243, Jan. 13, 1989, pp. 220-222, J. A. Zwiebel, et al., "High-Level Recombinant Gene Ex-

pression in Rabbit Endothelial Cells Transduced by Retroviral Vectors".

Science, vol. 244, Jun. 16, 1989, pp. 1342-1344, E. G. Nabel, et al., "Recombinant Gene Expression in Vivo Within Endothelial Cells of the Arterial Wall".

Science, vol. 236, May 8, 1987, pp. 714-718, R. F. Seldon, et al., "Implantation of Genetically Engineered Fibroblasts Into Mice: Implications for Gene Therapy". Proc. Nat. Acad. Sci. USA, vol. 84, pp. 7413-7417, No. 16, 1987, P. L. Felgner, et al., "Lipofection: A Highly Efficient, Lipid-Mediated DNA-Transfection Procedure".

Science, vol. 244, Jun. 16, 1989, pp. 1344-1346, J. M. Wilson, et al., "Implantation of Vascular Grafts Lined With Genetically Modified Endothelial Cells".

Federation of American Societies for Experimental Biology Journal, vol. 5, No. 4, pp. 539A, K. N. Bradley, et al., "Transfection of Wounds in vivo With Transforming Growth Factor- β_1 Accelerates Healing".

Cancer Research, vol. 50, Aug. 15, 1990, pp. 5102-5106, H. Ogura, et al., "Implantation of Genetically Manipulated Fibroblasts Into Mice as Antitumor α -Interferon Therapy".

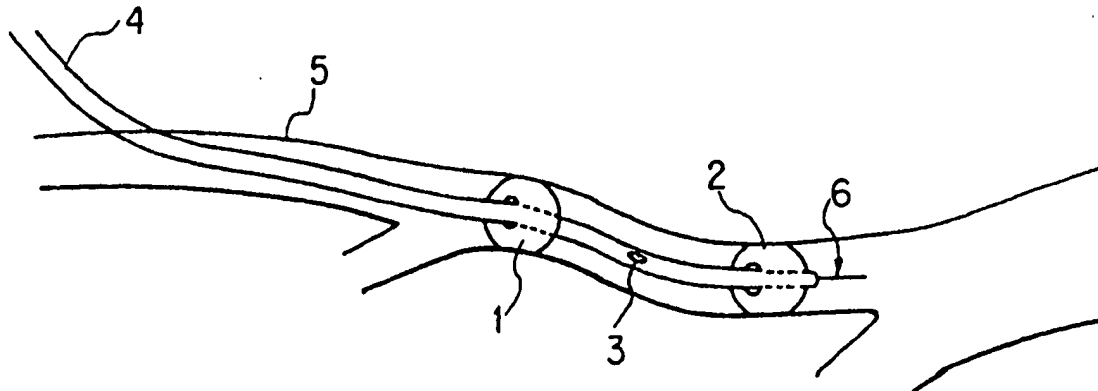
Primary Examiner—C. Fred Rosenbaum

Assistant Examiner—V. Alexander

Attorney, Agent, or Firm—Obolon, Spivak, McClelland, Maier & Neustadt

[57]**ABSTRACT**

A method for the direct treatment towards the specific sites of a disease is disclosed. This method is based on the delivery of proteins by catheterization to discrete blood vessel segments using genetically modified or normal cells or other vector systems. Endothelial cells expressing recombinant therapeutic agent or diagnostic proteins are situated on the walls of the blood vessel or in the tissue perfused by the vessel in a patient. This technique, provides for the transfer of cells or vectors and expression of recombinant genes in vivo and allows the introduction of proteins of therapeutic or diagnostic value for the treatment of diseases.

10 Claims, 4 Drawing Sheets

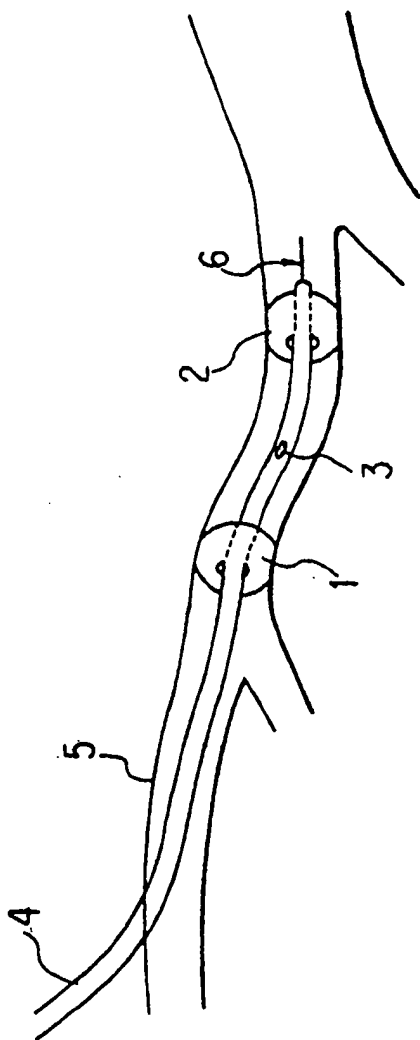


FIG. 1

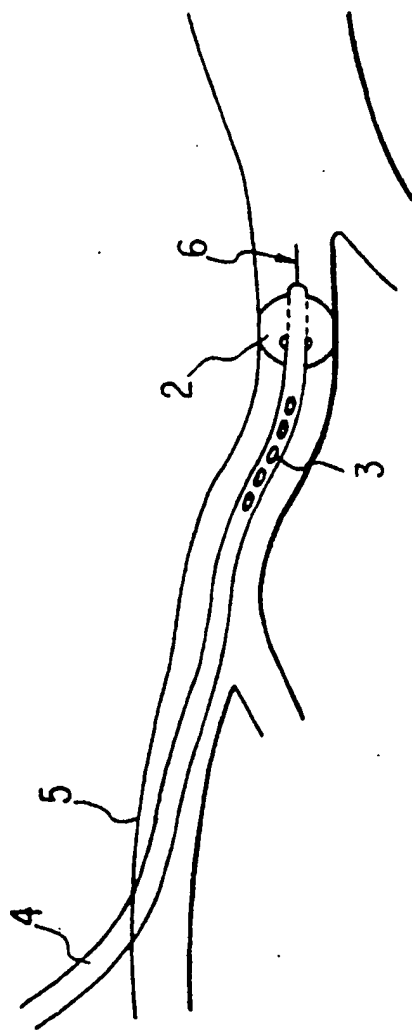
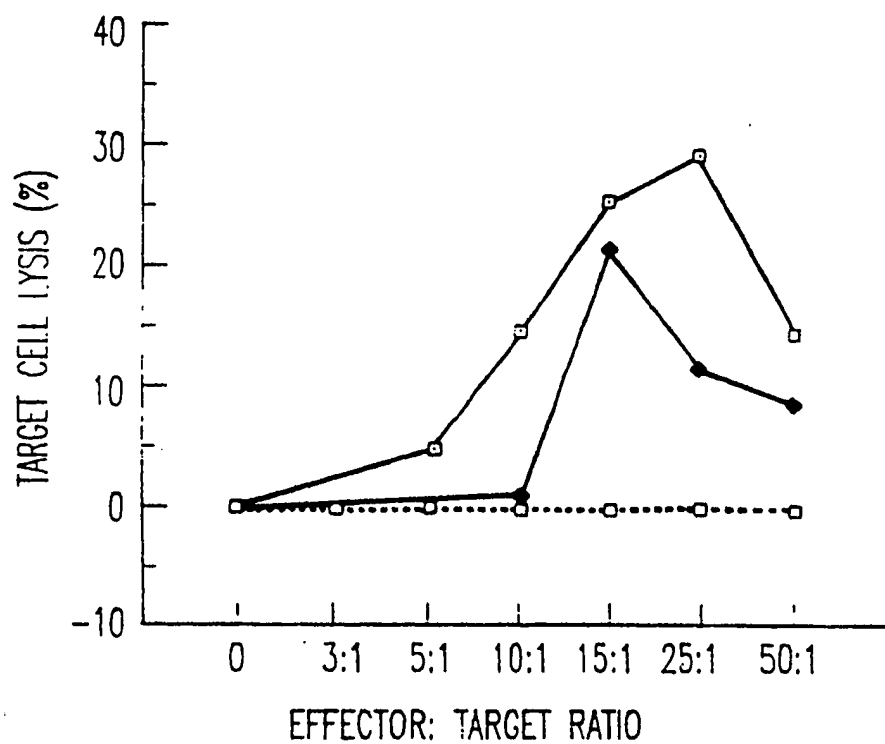


FIG. 2

FIG. 3I.V. INJECTIONS

□ — SOLUTION C/H-2Ks

◆ — LIPOFECTIN / H-2Ks

□ - - - LIPOFECTIN / RSV-B-GAL

WESTERN BLOT ANALYSIS

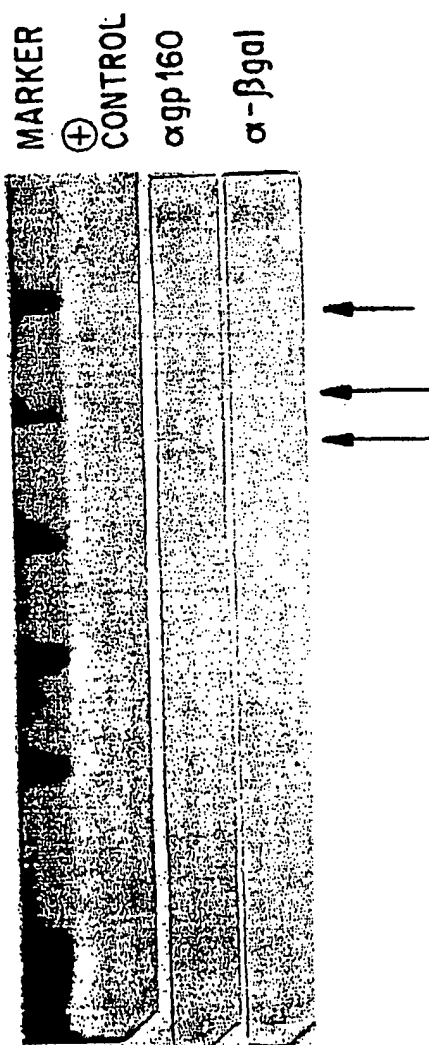


FIG. 4

TREATMENT OF DISEASES BY SITE-SPECIFIC INSTALLATION OF CELLS OR SITE-SPECIFIC TRANSFORMATION OF CELLS AND KITS THEREFOR

This is a continuation-in-part of U.S. patent application Ser. No. 07/724,509, filed on Jun. 28, 1991, now pending, which is a continuation-in-part of U.S. patent application Ser. No. 07/331,336, filed on Mar. 31, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the treatment of diseases by the site-specific instillation or transformation of cells and kits therefor. The present invention also relates to a method for modulating the immune system of an animal by the in vivo introduction of recombinant genes.

2. Discussion of the Background

The effective treatment of many acquired and inherited diseases remains a major challenge to modern medicine. The ability to deliver therapeutic agents to specific sites in vivo would be an asset in the treatment of, e.g., localized diseases. In addition the ability to cause a therapeutic agent to perfuse through the circulatory system would be effective for the treatment of, e.g., inherited diseases and acquired diseases or cancers.

For example, it would be desirable to administer in a steady fashion an antitumor agent or toxin in close proximity to a tumor. Similarly, it would be desirable to cause a perfusion of, e.g., insulin in the blood of a person suffering from diabetes. However, for many therapeutic agents there is no satisfactory method of either site-specific or systemic administration.

In addition, for many diseases, it would be desirable to cause, either locally or systemically, the expression of a defective endogenous gene, the expression of an exogenous gene, or the suppression of an endogenous gene. Again, these remain unrealized goals.

In particular, the pathogenesis of atherosclerosis is characterized by three fundamental biological processes. These are: 1) proliferation of intimal smooth muscle cells together with accumulated macrophages; 2) formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix; and 3) accumulation of lipid, principally in the form of cholesterol esters and free cholesterol, within cells as well as in surrounding connective tissue.

Endothelial cell injury is an initiating event and is manifested by interference with the permeability barrier of the endothelium, alterations in the nonthrombogenic properties of the endothelial surface, and promotion of procoagulant properties of the endothelium. Monocytes migrate between endothelial cells, become active as scavenger cells, and differentiate into macrophages.

Macrophages then synthesize and secrete growth factors including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF- α). These growth factors are extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells in the atherosclerotic plaque. In addition, platelets may interact with the injured endothelial cell and the activated macrophage to potentiate the elaboration of growth factors and thrombus formation.

Two major problems in the clinical management of coronary artery disease include thrombus formation in acute myocardial ischemia and restenosis following coronary angioplasty (PTCA). Both involve common cellular events, including endothelial injury and release of potent growth factors by activated macrophages and platelets. Coronary angioplasty produces fracturing of the atherosclerotic plaque and removal of the endothelium. This vascular trauma promotes platelet aggregation and thrombus formation at the PTCA site. Further release of mitogens from platelets and macrophages, smooth muscle cell proliferation and monocyte infiltration result in restenosis.

Empiric therapy with antiplatelet drugs has not prevented this problem, which occurs in one-third of patients undergoing PTCA. A solution to restenosis is to prevent platelet aggregation, thrombus formation, and smooth muscle cell proliferation.

Thrombus formation is also a critical cellular event in the transition from stable to unstable coronary syndromes. The pathogenesis most likely involves acute endothelial cell injury and/or plaque rupture, promoting dysjunction of endothelial cell attachment, and leading to the exposure of underlying macrophage foam cells. This permits the opportunity for circulating platelets to adhere, aggregate, and form thrombi.

The intravenous administration of thrombolytic agents, such as tissue plasminogen activator (tPA) results in lysis of thrombus in approximately 70% of patients experiencing an acute myocardial infarction. Nonetheless, approximately 30% of patients fail to reperfuse, and of those patients who undergo initial reperfusion of the infarct related artery, approximately 25% experience recurrent thrombosis within 24 hours. Therefore, an effective therapy for rethrombosis remains a major therapeutic challenge facing the medical community today.

As noted above, an effective therapy for rethrombosis is by far not the only major therapeutic challenge existing today. Others include the treatment of other ischemic conditions, including unstable angina, myocardial infarction or chronic tissue ischemia, or even the treatment of acquired and inherited diseases or cancers. These might be treated by the effective administration of anticoagulants, vasodilatory, angiogenic, growth factors or growth inhibitors to a patient. Thus, there remains a strongly felt need for an effective therapy in all of these clinical settings.

In addition, it is desirable to be able to modulate the immune system of an animal. In particular, much effort has been directed toward the development of vaccines to provide immunological protection from infection. However, the development of safe vaccines which can be readily administered to large numbers of patients is problematic, and for many diseases, such as, e.g., AIDS, no safe and effective vaccine is as yet available. Further, it is also sometimes desirable to specifically suppress an animal's immune response to prevent rejection of a transplant. Efforts to suppress transplant rejection have resulted in the development of drugs which result in a general suppression of the immune response, rather than specific suppression to transplantation antigens, and such drugs are not always effective. Thus, there remains a need for a method to modulate the immune system of an animal.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a novel method for the site-specific administration of a therapeutic agent.

It is another object of the present invention to provide a method for the perfusion of a therapeutic agent in the blood stream of a patient.

It is another object of the present invention to provide a method for causing the expression of an exogenous gene in a patient.

It is another object of the present invention to provide a method for causing the expression of a defective endogenous gene in a patient.

It is another object of the present invention to provide a method for suppressing the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the site-specific administration of a therapeutic agent or the perfusion of a therapeutic agent in the bloodstream of a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the expression of an exogenous gene, the expression of a defective endogenous gene, or the suppression of the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a kit for site-specifically instilling normal or transformed cells in a patient.

It is another object of the present invention to provide a kit for site-specifically transforming cells in vivo.

It is another object of the present invention to provide a method for modulating the immune system of an animal.

It is another object of the present invention to provide a method for modulating the immune system of an animal to sensitize the animal to a foreign molecule.

It is another object of the present invention to provide a method to stimulate the immune system of an animal to reject proteins in order to protect against infection by a microorganism or virus.

It is another object of the present invention to provide a method for modulating the immune system of an animal to tolerize the animal to a foreign molecule.

It is another object of the present invention to provide a method for modulating the immune system of an animal to reduce the tendency to reject a transplant.

It is another object of the present invention to provide a novel kit for transforming cells by systemic administration in vivo.

These and other objects of this invention which will become apparent during the course of the following detailed description of the invention have been discovered by the inventors to be achieved by (a) a method which comprises either (i) site-specific instillation of either normal (untransformed) or transformed cells in a patient or (ii) site-specific transformation of cells in a patient and (b) a kit which contains a catheter for (i) site-specific instillation of either normal or transformed cells or (ii) site-specific transformation of cells.

Site-specific instillation of normal cells can be used to replace damaged cells, while instillation of transformed cells can be used to cause the expression of either a defective endogenous gene or an exogenous gene or the suppression of an endogenous gene product. Instillation of cells in the walls of the patient's blood vessels can be used to cause the steady perfusion of a therapeutic agent in the blood stream.

The inventors have also discovered that by transforming cells of an animal, in vivo, it is possible to modulate the animal's immune system. In particular, by transforming cells of an animal, with a recombinant gene, by site-specific or systemic administration it is possible to modulate the animal's immune system to sensitize the animal to the molecule for which the recombinant gene encodes. Alternatively, by transforming cells of an animal with a recombinant gene, specifically at a site which determines the specificity of the immune system, such as, e.g., the thymus, it is possible to modulate the immune system of an animal to suppress the immune response to the molecule encoded by the recombinant gene.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures, wherein:

FIGS. 1 and 2 illustrate the use of a catheter in accordance with the invention to surgically or percutaneously implant cells in a blood vessel or to transform in vivo cells present on the wall of a patient's blood vessel;

FIG. 3 illustrates the relationship between the % of target cell lysis and the effector:target ratio for CTL cells; and

FIG. 4 illustrates the results of a Western blot analysis.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Thus, in one embodiment, the present invention is used to treat diseases, such as inherited diseases, systemic diseases, diseases of the cardiovascular system, diseases of particular organs, or tumors by instilling normal or transformed cells or by transforming cells.

The cells which may be instilled in the present method include endothelium, smooth muscle, fibroblasts, monocytes, macrophages, and parenchymal cells. These cells may produce proteins which may have a therapeutic or diagnostic effect and which may be naturally occurring or arise from recombinant genetic material.

Referring now to the figures, wherein like reference numerals designate identical or corresponding parts throughout the several views, and more particularly to FIG. 1 thereof, this figure illustrates the practice of the present invention with a catheter having a design as disclosed in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference. This catheter may be used to provide normal or genetically altered cells on the walls of a vessel or to introduce vectors for the local transformation of cells. In the figure, 5 is the wall of the blood vessel. The figure shows the catheter body 4 held in place by the inflation of inflatable balloon means 1 and 2. The section of the catheter body 4 situated between balloon means 1 and 2 is equipped with instillation port

means 3. The catheter may be further equipped with a guidewire means 6. FIG. 2 illustrates the use of a similar catheter, distinguished from the catheter illustrated in FIG. 1 by the fact that it is equipped with only a single inflatable balloon means 2 and a plurality of instillation port means 3. This catheter may contain up to twelve individual instillation port means 3, with five being illustrated.

In the case of delivery to an organ, the catheter may be introduced into the major artery supplying the tissue. Cells containing recombinant genes or vectors can be introduced through a central instillation port after temporary occlusion of the arterial circulation. In this way, cells or vector DNA may be delivered to a large amount of parenchymal tissue distributed through the capillary circulation. Recombinant genes can also be introduced into the vasculature using the double balloon catheter technique in the arterial circulation proximal to the target organ. In this way, the recombinant genes may be secreted directly into the circulation which perfuse the involved tissue or may be synthesized directly within the organ.

In one embodiment, the therapeutic agents are secreted by vascular cells supplying specific organs affected by the disease. For example, ischemic cardiomyopathy may be treated by introducing angiogenic factors into the coronary circulation. This approach may also be used for peripheral, vascular or cerebrovascular diseases where angiogenic factors may improve circulation to the brain or other tissues. Diabetes mellitus may be treated by introduction of glucose-responsive insulin secreting cells in the portal circulation where the liver normally sees a higher insulin concentration than other tissues.

In addition to providing local concentrations of therapeutic agents, the present method may also be used for delivery of recombinant genes to parenchymal tissues, because high concentrations of viral vector and other vectors can be delivered to a specific circulation. Using this approach, deficiencies of organ-specific proteins may also be treated. For example, in the liver, α -antitrypsin inhibitor deficiency or hypercholesterolemia may be treated by introduction of α -antitrypsin or the LDL receptor gene. In addition, this approach may be used for the treatment of a malignancy. Secretion of specific recombinant toxin genes into the circulation of inoperable tumors provides a therapeutic effect. Examples include acoustic neuromas or certain hemangiomas which are otherwise unresectable.

In clinical settings, these therapeutic recombinant genes are introduced in cells supplying the circulation of the involved organ. Although the arterial and capillary circulations are the preferred locations for introduction of these cells, venous systems are also suitable.

In its application to the treatment of local vascular damage the present invention provides for the expression of proteins which ameliorate this condition in situ. In one embodiment, because vascular cells are found at these sites, they are used as carriers to convey the therapeutic agents.

The invention thus, in one of its aspects, relies on genetic alteration of endothelial and other vascular cells or somatic cell gene therapy, for transmitting therapeutic agents (i.e., proteins, growth factors) to the localized region of vessel injury. To successfully use gene transplantation in the cells, four requirements must be fulfilled. First, the gene which is to be implanted into the cell must be identified and isolated. Second, the gene to

be expressed must be cloned and available for genetic manipulation. Third, the gene must be introduced into the cell in a form that will be expressed or functional. Fourth, the genetically altered cells must be situated in the vascular region where it is needed.

In accordance with the present invention the altered cells or appropriate vector may be surgically, percutaneously, or intravenously introduced and attached to a section of a patient's vessel wall. Alternatively, some of the cells existing on the patient's vessel wall are transformed with the desired genetic material or by directly applying the vector. In some instances, vascular cells which are not genetically modified can be introduced by these methods to replace cells lost or damaged on the vessel surface.

Any blood vessel may be treated in accordance with this invention; that is, arteries, veins, and capillaries. These blood vessels may be in or near any organ in the human, or mammalian, body.

Introduction of normal or genetically altered cells into a blood vessel

This embodiment of the invention may be illustrated as follows:

I. Establishment of endothelial or other vascular cells in tissue culture.

Initially, a cell line is established and stored in liquid nitrogen. Prior to cryopreservation, an aliquot is taken for infection or transfection with a vector, viral or otherwise, containing the desired genetic material.

Endothelial or other vascular cells may be derived enzymatically from a segment of a blood vessel, using techniques previously described in J. W. Ford, et al., *In Vitro*, 17, 40 (1981). The vessel is excised, inverted over a stainless steel rod and incubated in 0.1% trypsin in Ca^{++} and Mg^{++} free Hank's balanced salt solution (BSS) with 0.125% EDTA at pH 8 for 10 min at 37° C.

Cells (0.4 to 1.5×10^6) are collected by centrifugation and resuspended in medium 199 (GIBCO) containing 10% fetal bovine serum, endothelial cell growth supplement (ECGS, Collaborative Research, Waltham, Mass.) at 25 $\mu\text{g}/\text{ml}$, heparin at 15 U/ml, and gentamicin (50 $\mu\text{g}/\text{ml}$). Cells are added to a 75 cm^2 tissue culture flask precoated with gelatin (2 mg/ml in distilled water). Cells are fed every second day in the above medium until they reach confluence.

After two weeks in culture, the ECGS and heparin may be omitted from the medium when culturing porcine endothelium. If vascular smooth muscle cells or fibroblasts are desired the heparin and ECGS can be omitted entirely from the culturing procedure. Aliquots of cells are stored in liquid nitrogen by resuspending to approximately 10^6 cells in 0.5 ml of ice cold fetal calf serum on ice. An equal volume of ice cold fetal calf serum containing 10% DMSO is added, and cells are transferred to a prechilled screw cap Corning freezing tube. These cells are transferred to a -70°C freezer for 3 hours before long term storage in liquid nitrogen.

The cells are then infected with a vector containing the desired genetic material.

II. Introduction of cells expressing normal or exogenous proteins into the vasculature.

A. Introduction of cells expressing relevant proteins by catheterization.

The patient is prepared for catheterization either by surgery or percutaneously, observing strict adherence to sterile techniques. A cutdown procedure is performed over the target blood vessel or a needle is in-

serted into the target blood vessel after appropriate anesthesia. The vessel (5) is punctured and a catheter, such as described in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference. (available from USCI, Billerica, Mass.) is advanced by guidewire means (6) under fluoroscopic guidance, if necessary, into the vessel (5) (FIG. 1). This catheter means (4) is designed to introduce infected endothelial cells into a discrete region of the artery. The catheter has a proximal and distal balloon means (2) and (1), respectively, (e.g., each balloon means may be about 3 mm in length and about 4 mm in width), with a length of catheter means between the balloons. The length of catheter means between the balloons has a port means connected to an instillation port means (3). When the proximal and distal balloons are inflated, a central space is created in the vessel, allowing for instillation of infected cells through the port.

A region of the blood vessel is identified by anatomical landmarks and the proximal balloon means (2) is inflated to denude the endothelium by mechanical trauma (e.g., by forceful passage of a partially inflated balloon catheter within the vessel) or by mechanical trauma in combination with small amounts of a proteolytic enzyme such as dispase, trypsin, collagenase, papain, pepsin, chymotrypsin or cathepsin, or by incubation with these proteolytic enzymes alone. In addition to proteolytic enzymes, lipases may be used. The region of the blood vessel may also be denuded by treatment with a mild detergent or the like, such as NP-40, Triton X100, deoxycholate, or SDS.

The denudation conditions are adjusted to achieve essentially complete loss of endothelium for cell transfers or approximately 20 to 90%, preferably 50 to 75%, loss of cells from the vessel wall for direct infection. In some instances cell removal may not be necessary. The catheter is then advanced so that the instillation port means (3) is placed in the region of denuded endothelium. Infected, transfected or normal cells are then instilled into the discrete section of artery over thirty minutes. If the blood vessel is perfusing an organ which can tolerate some ischemia, e.g., skeletal muscle, distal perfusion is not a major problem, but can be restored by an external shunt if necessary, or by using a catheter which allows distal perfusion. After instillation of the infected endothelial cells, the balloon catheter is removed, and the arterial puncture site and local skin incision are repaired. If distal perfusion is necessary, an alternative catheter designed to allow distal perfusion may be used.

B. Introduction of recombinant genes directly into cells on the wall of a blood vessel or perfused by a specific circulation in vivo; infection or transfection of cells on the vessel wall and organs.

Surgical techniques are used as described above. Instead of using infected cells, a high titer desired genetic material transducing viral vector (10^5 to 10^6 particles/ml) or DNA complexed to a delivery vector is directly instilled into the vessel wall using the double balloon catheter technique. This vector is instilled in medium containing serum and polybrene (10 μ g/ml) to enhance the efficiency of infection. After incubation in the dead space created by the catheter for an adequate period of time (0.2 to 2 hours or greater), this medium is evacuated, gently washed with phosphate-buffered saline, and arterial circulation is restored. Similar protocols are used for post operative recovery.

The vessel surface can be prepared by mechanical denudation alone, in combination with small amounts of proteolytic enzymes such as dispase, trypsin, collagenase or cathepsin, or by incubation with these proteolytic enzymes alone. The denudation conditions are adjusted to achieve the appropriate loss of cells from the vessel wall.

Viral vector or DNA-vector complex is instilled in Dulbecco's modified Eagle's medium using purified virus or complexes containing autologous serum, and adhesive molecules such as polybrene (10 μ g/ml), poly-L-lysine, dextran sulfate, or any polycationic substance which is physiologically suitable, or a hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target in the vessel wall or in the tissue perfused by the vessel to enhance the efficiency of infection by increasing adhesion of viral particles to the relevant target cells. The hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target cell can be made by one of two methods. Antibodies directed against different epitopes can be chemically crosslinked (G. Jung, C. J. Honsik, R. A. Reisfeld, and H. J. Muller-Eberhard, *Proc. Natl. Acad. Sci. USA*, 83, 4479 (1986); U. D. Staerz, O. Kanagawa, and M. J. Bevan, *Nature*, 314, 628 (1985); and P. Perez, R. W. Hoffman, J. A. Titus, and D. M. Segal, *J. Exp. Med.*, 163, 166 (1986)) or biologically coupled using hybrid hybridomas (U. D. Staerz and M. J. Bevan, *Proc. Natl. Acad. Sci. USA*, 83, 1453 (1986); and C. Milstein and A. C. Cuellar, *Nature*, 305, 537 (1983)). After incubation in the central space of the catheter for 0.2 to 2 hours or more, the medium is evacuated, gently washed with phosphate buffered saline, and circulation restored.

Using a different catheter design (see FIG. 2), a different protocol for instillation can also be used. This second approach involves the use of a single balloon means (2) catheter with multiple port means (3) which allow for high pressure delivery of the retrovirus into partially denuded arterial segments. The vessel surface is prepared as described above and defective vector is introduced using similar adhesive molecules. In this instance, the use of a high pressure delivery system serves to optimize the interaction of vectors with cells in adjacent vascular tissue.

The present invention also provides for the use of growth factors delivered locally by catheter or systemically to enhance the efficiency of infection. In addition to retroviral vectors, herpes virus, adenovirus, or other viral vectors are suitable vectors for the present technique.

It is also possible to transform cells within an organ or tissue. Direct transformation of organ or tissue cells may be accomplished by one of two methods. In a first method a high pressure transfection is used. The high pressure will cause the vector to migrate through the blood vessel walls into the surrounding tissue. In a second method, injection into a capillary bed, optionally after injury to allow leaking, gives rise to direct infection of the surrounding tissues.

The time required for the instillation of the vectors or cells will depend on the particular aspect of the invention being employed. Thus, for instilling cells or vectors in a blood vessel a suitable time would be from 0.01 to 12 hrs, preferably 0.1 to 6 hrs, most preferably 0.2 to 2 hrs. Alternatively for high pressure instillation of vectors or cells, shorter times might be preferred.

Obtaining the cells used in this invention

The term "genetic material" generally refers to DNA which codes for a protein. This term also encompasses RNA when used with an RNA virus or other vector based on RNA.

Transformation is the process by which cells have incorporated an exogenous gene by direct infection, transfection or other means of uptake.

The term "vector" is well understood and is synonymous with the often-used phrase "cloning vehicle". A vector is non-chromosomal double-stranded DNA comprising an intact replicon such that the vector is replicated when placed within a unicellular organism, for example by a process of transformation. Viral vectors include retroviruses, adenoviruses, herpesvirus, papovirus, or otherwise modified naturally occurring viruses. Vector also means a formulation of DNA with a chemical or substance which allows uptake by cells.

In another embodiment the present invention provides for inhibiting the expression of a gene. Four approaches may be utilized to accomplish this goal. These include the use of antisense agents, either synthetic oligonucleotides which are complementary to the mRNA (Maher III, L. J. and Dolnick, B. J. *Arch. Biochem. Biophys.*, 253, 214-220 (1987) and Zamecnik, P. C., et al., *Proc. Natl. Acad. Sci.*, 83, 4143-4146 (1986)), or the use of plasmids expressing the reverse complement of this gene (Izant, J. H. and Weintraub, H., *Science*, 229, 345-352, (1985); *Cell*, 36, 1077-1015 (1984)). In addition, catalytic RNAs, called ribozymes, can specifically degrade RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596-600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585-591 (1988)). The third approach involves "intracellular immunization", where analogues of intracellular proteins can interfere specifically with their function (Friedman, A. D., Triezenberg, S. J. and McKnight, S. L., *Nature*, 335, 452-454 (1988)), described in detail below.

The first approaches may be used to specifically eliminate transcripts in cells. The loss of transcript may be confirmed by S1 nuclease analysis, and expression of binding protein determined using a functional assay. Single-stranded oligonucleotide analogues may be used to interfere with the processing or translation of the transcription factor mRNA. Briefly, synthetic oligonucleotides or thiol-derivative analogues (20-50 nucleotides) complementary to the coding strand of the target gene may be prepared. These antisense agents may be prepared against different regions of the mRNA. They are complementary to the 5' untranslated region, the translational initiation site and subsequent 20-50 base pairs, the central coding region, or the 3' untranslated region of the gene. The antisense agents may be incubated with cells transfected prior to activation. The efficacy of antisense competitors directed at different portions of the messenger RNA may be compared to determine whether specific regions may be more effective in preventing the expression of these genes.

RNA can also function in an autocatalytic fashion to cause autolysis or to specifically degrade complementary RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596-600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585-591 (1988), and Hutchins, C. J., et al, *Nucleic Acids Res.*, 14, 3627-3640 (1986)). The requirements for a successful RNA cleavage include a hammerhead structure with conserved RNA sequence at the region flanking this structure. Regions adjacent to this cata-

lytic domain are made complementary to a specific RNA, thus targeting the ribozyme to specific cellular mRNAs. To inhibit the production of a specific target gene, the mRNA encoding this gene may be specifically degraded using ribozymes. Briefly, any GUG sequence within the RNA transcript can serve as a target for degradation by the ribozyme. These may be identified by DNA sequence analysis and GUG sites spanning the RNA transcript may be used for specific degradation. Sites in the 5' untranslated region, in the coding region, and in the 3' untranslated region may be targeted to determine whether one region is more efficient in degrading this transcript. Synthetic oligonucleotides encoding 20 base pairs of complementary sequence upstream of the GUG site, the hammerhead structure and ~20 base pairs of complementary sequence downstream of this site may be inserted at the relevant site in the cDNA. In this way, the ribozyme may be targeted to the same cellular compartment as the endogenous message. The ribozymes inserted downstream of specific enhancers, which give high level expression in specific cells may also be generated. These plasmids may be introduced into relevant target cells using electroporation and cotransfection with a neomycin resistant plasmid, pSV2-Neo or another selectable marker. The expression of these transcripts may be confirmed by Northern blot and S1 nuclease analysis. When confirmed, the expression of mRNA may be evaluated by S1 nuclease protection to determine whether expression of these transcripts reduces steady state levels of the target mRNA and the genes which it regulates. The level of protein may also be examined.

Genes may also be inhibited by preparing mutant transcripts lacking domains required for activation. Briefly, after the domain has been identified, a mutant form which is incapable of stimulating function is synthesized. This truncated gene product may be inserted downstream of the SV-40 enhancer in a plasmid containing the neomycin resistance gene (Mulligan, R. and Berg, P., *Science*, 209, 1422-1427 (1980) (in a separate transcription unit). This plasmid may be introduced into cells and selected using G418. The presence of the mutant form of this gene will be confirmed by S1 nuclease analysis and by immunoprecipitation. The function of the endogenous protein in these cells may be evaluated in two ways. First, the expression of the normal gene may be examined. Second, the known function of these proteins may be evaluated. In the event that this mutant intercellular interfering form is toxic to its host cell, it may be introduced on an inducible control element, such as metallothionein promoter. After the isolation of stable lines, cells may be incubated with Zn or Cd to express this gene. Its effect on host cells can then be evaluated.

Another approach to the inactivation of specific genes is to overexpress recombinant proteins which antagonize the expression or function of other activities. For example, if one wished to decrease expression of TPA (e.g., in a clinical setting of disseminate thrombolysis), one could overexpress plasminogen activator inhibitor.

Advances in biochemistry and molecular biology in recent years have led to the construction of "recombinant" vectors in which, for example, retroviruses and plasmids are made to contain exogenous RNA or DNA, respectively. In particular instances the recombinant vector can include heterologous RNA or DNA, by which is meant RNA or DNA that codes for a polypep-

tide ordinarily not produced by the organism susceptible to transformation by the recombinant vector. The production of recombinant RNA and DNA vectors is well understood and need not be described in detail. However, a brief description of this process is included here for reference.

For example, a retrovirus or a plasmid vector can be cleaved to provide linear RNA or DNA having ligatable termini. These termini are bound to exogenous RNA or DNA having complementary like ligatable termini to provide a biologically functional recombinant RNA or DNA molecule having an intact replicon and a desired phenotypical property.

A variety of techniques are available for RNA and DNA recombination in which adjoining ends of separate RNA or DNA fragments are tailored to facilitate ligation.

The exogenous, i.e., donor, RNA or DNA used in the present invention is obtained from suitable cells. The vector is constructed using known techniques to obtain a transformed cell capable of in vivo expression of the therapeutic agent protein. The transformed cell is obtained by contacting a target cell with a RNA- or DNA-containing formulation permitting transfer and uptake of the RNA or DNA into the target cell. Such formulations include, for example, retroviruses, plasmids, liposomal formulations, or plasmids complexes with polycationic substances such as poly-L-lysine, DEAC-dextran and targeting ligands.

The present invention thus provides for the genetic alteration of cells as a method to transmit therapeutic or diagnostic agents to localized regions of the blood vessel for local or systemic purposes. The range of recombinant proteins which may be expressed in these cells is broad and varied. It includes gene transfer using vectors expressing such proteins as tPA for the treatment of thrombosis and restenosis, angiogenesis or growth factors for the purpose of revascularization, and vasoactive factors to alleviate vasoconstriction or vasospasm. This technique can also be extended to genetic treatment of inherited disorders, or acquired diseases, localized or systemic. The present invention may also be used to introduce normal cells to specific sites of cell loss, for example, to replace endothelium damaged during angioplasty or catheterization.

For example, in the treatment of ischemic diseases (thrombotic diseases), genetic material coding for tPA or modifications thereof, urokinase or streptokinase is used to transform the cells. In the treatment of ischemic organ (e.g., heart, kidney, bowel, liver, etc.) failure, genetic material coding for recollateralization agents, such as transforming growth factor α (TGF- α), transforming growth factor β (TGF- β) angiogenin, tumor necrosis factor α , tumor necrosis factor β , acidic fibroblast growth factor or basic fibroblast growth factor can be used. In the treatment of vasomotor diseases, genetic material coding for vasodilators or vasoconstrictors may be used. These include atrial natriuretic factor, platelet-derived growth factor or endothelin. In the treatment of diabetes, genetic material coding for insulin may be used.

The present invention can also be used in the treatment of malignancies by placing the transformed cells in proximity to the malignancy. In this application, genetic material coding for diphtheria toxin, pertussis toxin, or cholera toxin may be used.

In one of its embodiments, the present invention provides for the therapy of malignancy by either stimulat-

ing an immune response against tumor cells or inhibiting tumor cell growth or metastasis by genetic modification in vivo. This approach differs from previous methods in which tumor cells are propagated, modified, and selected in vitro.

In accordance with this embodiment, the present method is used to deliver a DNA sequence or an RNA sequence, including recombinant genes, to tumor cells in vivo with (1) retroviral or viral vectors as vehicles, (2) DNA or RNA/liposome complexes as vehicles, (3) chemical formulations containing the DNA or RNA sequence and coupled to a carrier molecule which facilitates delivery of the sequence to the targeted cells, or (4) by utilizing cell-mediated gene transfer to deliver genes to specific sites in vivo, e.g., by relying upon the use of vascular smooth muscle cells or endothelial cells which have been transduced in vitro as a vehicle to deliver the recombinant gene into the site of the tumor.

In an aspect of this embodiment, the present invention relies on the immune system to provide protection against cancer and play an important role as an adjuvant treatment for a malignancy. Immunotherapy has shown promise as an adjuvant approach to the treatment of malignancies. Both cytolytic T cells and lymphokines can facilitate tumor cell destruction, and strategies to enhance tumor regression by administration of cytokines or tumor infiltrating lymphocytes have shown efficacy in animal models and human trials. For example, it is known that lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) can lyse neoplastic cells and produce partial or complete tumor rejection. Expression of cytokine genes in malignant cells has also enhanced tumor regression.

The present invention provides a novel gene transfer approach against tumors by the introduction of recombinant genes directly into tumor cells in vivo, where, by contrast, traditional gene transfer techniques have focused on modification of tumor cells in vitro followed by transfer of the modified cells. The prior art approaches are disadvantageous because they subject the cells to selection in different growth conditions from those which act in vivo, and because they also require that cell lines be established for each malignancy, thereby rendering adaptability to human disease considerably more difficult.

Genes which may be used with this embodiment include genes containing a DNA sequence (or the corresponding RNA sequence may be used) encoding an intracellular, secreted, or cell surface molecule which is exogenous to the patient and which (1) is immunogenic to the patient, (2) induces rejection, regression, or both, of the tumor, or (3) is toxic to the cells of the tumor.

The vectors containing the DNA sequence (or the corresponding RNA sequence) which may be used in accordance with the invention may be an eukaryotic expression vector containing the DNA or the RNA sequence of interest. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman et al, *Proc. Nat. Acad. Sci. (USA)*, (1987) 84:2150-2154, which is hereby incorporated by reference.

This vector, as noted above, may be administered to the patient in a retroviral or other viral vector (i.e., a viral vector) vehicle, a DNA or RNA/liposome complex, or by utilizing cell-mediated gene transfer. Further, the vector, when present in non-viral form, may be administered as a DNA or RNA sequence-containing chemical formulation coupled to a carrier molecule

which facilitates delivery to the host cell. Such carrier molecule would include an antibody specific to the cells to which the vector is being delivered or a molecule capable of interacting with a receptor associated with the target cells.

Cell-mediated gene transfer may be used in accordance with the invention. In this mode, one relies upon the delivery of recombinant genes into living organisms by transfer of the genetic material into cells derived from the host and modification in cell culture, followed by the introduction of genetically altered cells into the host. An illustrative packaging cell line which may be used in accordance with this embodiment is described in Danos et al, *Proc. Natl. Acad. Sci. (USA)* (1988) 85:6460, which is hereby incorporated by reference.

The DNA or RNA sequence encoding the molecule used in accordance with the invention may be administered to the patient, which may be human or a non-human animal, either locally or systemically. The systemic administration is preferably carried out using the non-viral DNA or RNA chemical formulation coupled to a carrier molecule which facilitates delivery to the host cells. Any of the administrations may be performed by IV or IM injection or subcutaneous injection using any known means, or by the use of the catheter in accordance with the present invention.

The retroviral vector vehicles used in accordance with the present invention comprise a viral particle derived from a naturally-occurring retrovirus which has been genetically altered to render it replication defective and to express a recombinant gene of interest in accordance with the invention. Once the virus delivers its genetic material to a cell, it does not generate additional infectious virus but does introduce exogenous recombinant genes to the cell.

In other viral vectors, the virus particle used is derived from other naturally-occurring viruses which have been genetically altered to render them replication defective and to express recombinant genes. Such viral vectors may be derived from adenovirus, papillomavirus, herpesvirus, parvovirus, etc.

The sequences of the present invention may also be administered as DNA or RNA/liposome complex. Such complexes comprise a mixture of fat particles, lipids, which bind to genetic material, DNA or RNA, providing a hydrophobic coat, allowing genetic material to be delivered into cells. This formulation provides a non-viral vector for gene transfer. Liposomes used in accordance with the invention may comprise DOPE (dioleoyl phosphatidyl ethanol amine), CUDMEDA (N-(5-cholestrum-3- β -ol 3-urethanyl)-N',N'-dimethyl-ethylene diamine).

As noted above, other non-viral vectors may also be used in accordance with the present invention. These include chemical formulations of DNA or RNA coupled to a carrier molecule (e.g., an antibody or a receptor ligand) which facilitates delivery to host cells for the purpose of altering the biologic properties of the host cells. The term "chemical formulations" used herein refers to modifications of nucleic acids to allow coupling of the nucleic acid compounds to a protein or lipid, or derivative thereof, carrier molecule. Such carrier molecules include antibodies specific to the host cells or receptor ligands, i.e., molecules able to interact with receptors associated with the host cells.

The molecules which may be used in accordance with this invention, include the following: (1) genes encoding immune stimulants, such as Class I histocom-

patibility genes, Class II histocompatibility genes, bacterial genes, including mycobacterial (PPD) genes and genes encoding heat shock proteins, viral glycoproteins encoding genes, including vesicular stomatitis virus G protein, influenza hemagglutinin, and herpes virus glycoprotein β , minor histocompatibility antigens, foreign proteins, such as lysozyme or bovine serum albumin, and oncogenes, including EIA, P53 (mutants) and tax; (2) immune and growth stimulants/inhibitors, including inducers of differentiation, such as stimulants, including interleukin-2 (IL-2) IL-4, 3, 6 or 8, inhibitors/inducers of differentiation, such as TNF- α or β , TGF- β (1, 2 or 3), IL-1, soluble growth factor receptors (PDGF, FGF receptors), recombinant antibodies to growth factors or receptors, analogs of growth factors (PDGF, FGF), interferons (α , β or γ) and adhesion molecules; or (3) toxins or negative selectable markers, including thymidine kinase, diphtheria toxin, pertussis toxin or drug-sensitive proteins.

The DNA/RNA sequence is preferably obtained from a source of the same species as the patient, but this is not absolutely required, and the present invention provides for the use of DNA sequences obtained from a source of a species different from the patient in accordance with this embodiment. A preferred embodiment of the present invention, genes encoding immune stimulants and toxins or negative selectable markers, corresponding to (1) and (3) above, are preferably selected from a species different than the species to which the patient belongs. For immune and growth stimulants/inhibitors, corresponding to (2) above, in accordance with another preferred embodiment of the invention, one preferably employs a gene obtained from a species which is the same as the species of the patient.

In the use of the present invention in the treatment of AIDS, genetic material coding for soluble CD4 or derivatives thereof may be used. In the treatment of genetic diseases, for example, growth hormone deficiency, genetic material coding for the needed substance, for example, human growth hormone, is used. All of these genetic materials are readily available to one skilled in this art.

In another embodiment, the present invention provides a kit for treating a disease in a patient which contains a catheter and a solution which contains either an enzyme or a mild detergent, in which the catheter is adapted for insertion into a blood vessel and contains a main catheter body having a balloon element adapted to be inserted into said vessel and expansible against the walls of the blood vessel so as to hold the main catheter body in place in the blood vessel, and means carried by the main catheter body for delivering a solution into the blood vessel, and the solution which contains the enzyme or mild detergent is a physiologically acceptable solution. The solution may contain a proteolytic enzyme, such as dispase, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, lipases may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Alternatively, the kit may contain a physiological acceptable solution which contains an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, or bivalent antibodies. This solution may also contain vectors or cells (normal or transformed). In yet another embodiment the kit may contain a catheter and both a solution which contains an enzyme or mild detergent and a solution which contains

an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material or bivalent antibody and which may optionally contain vectors or cells.

The kit may contain a catheter with a single balloon and central distal perfusion port, together with acceptable solutions to allow introduction of cells in a specific organ or vectors into a capillary bed or cells in a specific organ or tissue perfused by this capillary bed.

Alternatively, the kit may contain a main catheter body which has two spaced balloon elements adapted to be inserted in a blood vessel with both being expansible against the walls of the blood vessel for providing a chamber in the blood vessel, and to hold the main catheter body in place. In this case, the means for delivering a solution into the chamber is situated in between the balloon elements. The kit may contain a catheter which possesses a plurality of port means for delivering the solution into the blood vessel.

Thus, the present invention represents a method for treating a disease in a patient by causing a cell attached onto the walls of a vessel or the cells of an organ perfused by this vessel in the patient to express an exogenous therapeutic agent protein, wherein the protein treats the disease or may be useful for diagnostic purposes. The present method may be used to treat diseases, such as an ischemic disease, a vasomotor disease, diabetes, a malignancy, AIDS or a genetic disease.

The present method may use exogenous therapeutic agent proteins, such as tPA and modifications thereof, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α , tumor necrosis factor β , transforming growth factor α , transforming growth factor β , atrial natriuretic factor, platelet-derived growth factor, endothelial, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and derivatives thereof, and growth hormone to treat diseases.

The present method may also use exogenous proteins of diagnostic value. For example, a marker protein, such as β -galactosidase, may be used to monitor cell migration.

It is preferred, that the cells caused to express the exogenous therapeutic agent protein be endothelial cells.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The data reported below demonstrate the feasibility of endothelial cell transfer and gene transplantation; that endothelial cells may be stably implanted in situ on the arterial wall by catheterization and express a recombinant marker protein, β -galactosidase, in vivo.

Because atherogenesis in swine has similarities to humans, an inbred pig strain, the Yucatan minipig (Charles River Laboratories, Inc., Wilmington, Mass.), was chosen as an animal model (1). A primary endothelial cell line was established from the internal jugular vein of an 8 month-old female minipig. The endothelial cell identity of this line was confirmed in that the cells exhibited growth characteristics and morphology typical of porcine endothelium in tissue culture. Endothelial cells also express receptors for the acetylated form of low density lipoprotein (AcLDL), in contrast to fibroblasts and other mesenchymal cells (2). When analyzed for ACLDL receptor expression, greater than 99% of

the cultured cells contained this receptor, as judged by fluorescent ACLDL uptake.

Two independent β -galactosidase-expressing endothelial lines were isolated following infection with a murine amphotropic β -galactosidase-transducing retroviral vector (BAG), which is replication-defective and contains both β -galactosidase and neomycin resistance genes (3). Cells containing this vector were selected for their ability to grow in the presence of G-418. Greater than 90% of selected cells synthesized β -galactosidase by histochemical staining. The endothelial nature of these genetically altered cells was also confirmed by analysis of fluorescent ACLDL uptake. Infection by BAG retrovirus was further verified by Southern blot analysis which revealed the presence of intact proviral DNA at approximately one copy per genome.

Endothelial cells derived from this inbred strain, being syngeneic, were applicable for study in more than one minipig, and were tested in nine different experimental subjects. Under general anesthesia, the femoral and iliac arteries were exposed, and a catheter was introduced into the vessel (FIG. 1). Intimal tissues of the arterial wall were denuded mechanically by forceful passage of a partially inflated balloon catheter within the vessel. The artery was rinsed with heparinized saline and incubated with the neutral protease, dispase (50 U/ml), which removed any remaining luminal endothelial cells. Residual enzyme was rapidly inactivated by α 2 globulin in plasma upon deflating the catheter balloons and allowing blood to flow through the vessel segment. The cultured endothelial cells which expressed β -galactosidase were introduced using a specially designed arterial catheter (USCI, Billerica, Mass.) that contained two balloons and a central instillation port (FIG. 1).

When these balloons were inflated, a protected space was created within the artery into which cells were instilled through the central port 3 (FIG. 1). These endothelial cells, which expressed β -galactosidase, were allowed to incubate for 30 minutes to facilitate their attachment to the denuded vessel. The catheter was then removed, the arterial branch ligated, and the incision closed.

Segments of the artery inoculated with β -galactosidase-expressing endothelium were removed 2 to 4 weeks later. Gross examination of the arterial specimen after staining using the X-gal chromogen showed multiple areas of blue coloration, compared to an artery seeded with uninfected endothelium, indicative of β -galactosidase activity. Light microscopy documented β -galactosidase staining primarily in endothelial cells of the intima in experimentally seeded vessels.

In contrast, no evidence of similar staining was observed in control segments which had received endothelial cells containing no β -galactosidase. β -Galactosidase staining was occasionally evident in deeper intimal tissues, suggesting entrapment or migration of seeded endothelium within the previously injured vessel wall. Local thrombosis was observed in the first two experimental subjects. This complication was minimized in subsequent studies by administering acetylsalicylic acid prior to the endothelial cell transfer procedure and use of heparin anticoagulation at the time of inoculation. In instances of thrombus formation, β -galactosidase staining was seen in endothelial cells extending from the vessel wall to the surface of the thrombus.

A major concern of gene transplantation *in vivo* relates to the production of replication-competent retrovirus from genetically engineered cells. In these tests, this potential problem has been minimized through the use of a replication defective retrovirus. No helper virus was detectable among these lines after 20 passages *in vitro*. Although defective viruses were used because of their high rate of infectivity and their stable integration into the host cell genome (4), this approach to gene transfer is adaptable to other viral vectors.

A second concern involves the longevity of expression of recombinant genes *in vivo*. Endothelial cell expression of β -galactosidase appeared constant in vessels examined up to six weeks after introduction into the blood vessel in the present study.

These tests have demonstrated that genetically-altered endothelial cells can be introduced into the vascular wall of the Yucatan minipig by arterial catheterization. Thus, the present method can be used for the localized biochemical treatment of vascular disease using genetically-altered endothelium as a vector.

A major complication of current interventions for vascular disease, such as balloon angioplasty or insertion of a graft into a diseased vessel, is disruption of the atherosclerotic plaque and thrombus formation at sites of local tissue trauma (5). In part, this is mediated by endothelial cell injury (6). The present data show that genetically-altered endothelial cells can be introduced at the time of intervention to minimize local thrombosis.

This technique can also be used in other ischemic settings, including unstable angina or myocardial infarction. For instance, antithrombotic effects can be achieved by introducing cells expressing genes for tissue plasminogen activator or urokinase. This technology is also useful for the treatment of chronic tissue ischemia. For example, elaboration of angiogenic or growth factors (7) to stimulate the formation of collateral vessels to severely ischemic tissue, such as the myocardium. Finally, somatic gene replacement for systemic inherited diseases is feasible using modifications of this endothelial cell gene transfer technique.

Another aspect of the present invention relates a method for modulating the immune system of an animal by *in vivo* transformation of cells of the animal with a recombinant gene. The transformation may be carried out either in a non-site-specific or systemic manner or a site-specific manner. If the transformation is carried out in a systemic fashion or at sites other than those which confer specificity on the immune system, such as the thymus, then the immune system will be modulated to result in the animal being sensitized to the molecule for which the recombinant gene encodes. Alternatively, if the transformation is carried out in a site-specific manner and is localized to a site which determines the specificity of the immune system, e.g., the thymus, the immune system will be modulated to result in the animal being tolerized to the molecule encoded by the recombinant gene.

By the term sensitized, it is meant that the immune system exhibits a stronger response to the molecule encoded by the DNA after *in vivo* transformation as compared to before transformation. By the term tolerized, it is meant that the immune system displays a reduced response to the molecule encoded by the recombinant gene after transformation as compared to before transformation. Thus, one may modulate an immune system to provide either a resistance or a tolerance to the molecule encoded by the DNA.

Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as tumor antigens (carcinoembryonic antigen), protozoan antigens (pneumocystis), viral antigens (HIV gp120 and gp160, H. influenza antigen, and hepatitis B surface antigen), Lyme disease antigen, Bacterial antigens, and transplantation antigens (Class I or II), ras or other oncogenes, including erb-A or neu; cytoplasmic proteins, such as the raf oncogene, src oncogene, and abl oncogene; nuclear proteins, such as E1A oncogene, mutant p53 oncogene, tat, tax, rev, vpu, vpx, hepatitis core antigen, EBNA and viral genes; and secreted proteins, such as endotoxin, cholera toxin, TNF, and osteoclast activating factor.

Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as growth factor receptors, insulin receptors, thyroid hormone receptors, transplantation antigens (class I or II), blood group antigens, and LDL receptor; cytoplasmic proteins, such as cytochrome P450, galactosyl transferase, dystrophin, neomycin resistance gene, and bacterial heat shock protein; nuclear proteins, such as retinoblastoma and transdominant rev; and secreted proteins, such as growth hormone for dwarfs, insulin for diabetics, and adenosine deaminase.

It is to be understood that the nucleic acid, DNA, RNA, or derivative thereof, in the recombinant gene may be of any suitable origin. That is the nucleic acid may be isolated from a naturally occurring source or may be of synthetic origin.

The recombinant gene may be introduced in the cells of the animal using any conventional vector. Such vectors include viral vectors, cationic lipids complexed to DNA or RNA (DNA or RNA/liposomes) and DNA or RNA complexes with polycations, such as DEAE, dextran, and polybrene.

As noted above the recombinant gene can be introduced into cells in a site-specific manner to confer resistance to the molecule encoded by the recombinant gene. Suitable sites include, e.g., endothelial cells or reticuloendothelial cells in the vasculature or any specific tissue or organ. The form of the preparation containing the vector and recombinant gene used in the transformation will depend on the specific tissue to be transformed. Suitable preparations for transforming endothelial cells are described elsewhere in this specification. In addition, preparations suitable for oral or other means of administration (e.g., endoscopic) may be used to provide mucosal resistance. Such preparation could include detergents, gelatins, capsules or other delivery vehicles to protect against degradation and enhance delivery to the mucosal surface, in addition to the vector and gene.

Alternatively, the recombinant gene may be introduced in a site specific fashion to a site which determines the specificity of the immune system. The thymus is such a site (see: A. M. Posselt et al, Science, vol. 249, p. 1292 (1990)). Thus, by introducing a recombinant gene site-specifically into the thymus, the immune system may be modulated to result in a tolerance to the molecule encoded by the gene. In this way, transplant rejection may be suppressed. The same preparations and techniques used to site-specifically transform tumors described above may be used to introduce the recombinant gene into the thymus. Specifically, the transformation preparation may be injected directed into the thymus or tumor or into the vascular supply of the thymus or tumor.

The present method may be practiced on any animal, such as chickens or mammals such as cows, horses, cats, dogs, monkeys, lemurs or humans.

When the recombinant gene is introduced using a liposome, it is preferred to first determine in vitro the optimal values for the DNA: lipid ratios and the absolute concentrations of DNA and lipid as a function of cell death and transformation efficiency for the particular type of cell to be transformed and to use these values in the in vivo transformation. The in vitro determination of these values can be easily carried out using the techniques described in the Experimental Section of this specification.

Another aspect of the present invention relates to a kit for the in vivo systemic introduction of a recombinant gene into cells of an animal. Such a kit would include approximately the optimal amount of a carrier, such as a lipid, and nucleic acid, and/or a means of delivery, e.g., an endoscope or a syringe. The kit may also contain instructions for the administration of the transforming preparation. The carrier and nucleic acid may be freeze dried and may be packaged separately or premixed. The kit may also contain a solution to optimally reconstitute the complexes of the carrier and the nucleic acid, which provide for efficient delivery to cells in vivo. Such a solution may contain one or more ingredients, such as buffers, sugars, salts, proteins, and detergents.

Having generally described the invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Experimental section

A. Analysis of AcLDL receptor expression in normal and β -galactosidase-transduced porcine endothelial cells.

Endothelial cell cultures derived from the Yucatan minipig, two sublines infected with BAG retrovirus or 3T3 fibroblast controls were analyzed for expression of AcLDL receptor using fluorescent labelled AcLDL.

Endothelial cells were derived from external jugular veins using the neutral protease dispase (8). Excised vein segments were filled with dispase (50 U/ml in Hanks' balanced salt solution) and incubated at 30° C. for 20 minutes. Endothelium obtained by this means was maintained in medium 199 (GIBCO, Grand Island, N.Y.) supplemented with fetal calf serum (10%), 50 μ g/ml endothelial cell growth supplement (ECGS) and heparin (100 μ g/ml). These cells were infected with BAG retrovirus, and selected for resistance to G-418. Cell cultures were incubated with (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (DiI) AcLDL (Biomedical Technologies, Stoughton, Mass.) (10 μ g/ml) for 4-6 hrs. at 37° C., followed by three rinses with phosphate-buffered saline containing 0.5% glutaraldehyde. Cells were visualized by phase contrast and fluorescent microscopy.

B. Method of introduction of endothelial cells by catheterization.

A double balloon catheter was used for instillation of endothelial cells. The catheter has a proximal and distal balloon, each 6 mm in length and 5 mm in width, with a 20 mm length between the balloons. The central section of the catheter has a 2 mm pore connected to an instillation port. Proximal and distal balloon inflation isolates a central space, allowing for instillation of in-

fect cells through the port into a discrete segment of the vessel. For a schematic representation of cell introduction by catheter, see FIGS. 1 and 2.

Animal care was carried out in accordance with "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, Revised 1978). Female Yucatan minipigs (80-100 kg) were anesthetized with pentobarbital (20 mg/kg), intubated, and mechanically ventilated. These subjects underwent sterile surgical exposure of the iliac and femoral arteries. The distal femoral artery was punctured, and the double-balloon catheter was advanced by guidewire into the iliac artery. The external iliac artery was identified; the proximal balloon was partially inflated and passed proximally and distally so as to mechanically denude the endothelium. The catheter was then positioned with the central space located in the region of denuded endothelium, and both balloons were inflated. The denuded segment was irrigated with heparinized saline, and residual adherent cells were removed by instillation of dispase (20 U/ml) for 10 min. The denuded vessel was further irrigated with a heparin solution and the BAG-infected endothelial cells were instilled for 30 min. The balloon catheter was subsequently removed, and antegrade blood flow was restored. The vessel segments were excised 2 to 4 weeks later. A portion of the artery was placed in 0.5% glutaraldehyde for five minutes and stored in phosphate-buffered saline, and another portion was mounted in a paraffin block for sectioning. The presence of retroviral expressed β -galactosidase was determined by a standard histochemical technique (19).

C. Analysis of endothelial cells in vitro and in vivo.

β -Galactosidase activity was documented by histochemical staining in (A) primary endothelial cells from the Yucatan minipig, (B) a subline derived by infection with the BAG retroviral vector, (C) a segment of normal control artery, (D) a segment of artery instilled with endothelium infected with the BAG retroviral vector, (E) microscopic cross-section of normal control artery, and (F) microscopic cross-section of artery instilled with endothelium infected with the BAG retroviral vector.

Endothelial cells in tissue culture were fixed in 0.5% glutaraldehyde prior to histochemical staining. The enzymatic activity of the *E. coli* β -galactosidase protein was used to identify infected endothelial cells in vitro and in vivo. The β -galactosidase transducing Mo-MuLV vector (2), (BAG) was kindly provided by Dr. Constance Cepko. This vector used the wild type Mo-MuLV LTR as a promoter for the β -galactosidase gene. The simian virus 40 (SV-40) early promoter linked to the Tn5 neomycin resistance gene provides resistance to the drug G-418 and is inserted downstream of the β -galactosidase gene, providing a marker to select for retrovirus-containing, β -galactosidase expressing cells. This defective retrovirus was prepared from fibroblast ψ am cells (3,10), and maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% calf serum. Cells were passaged twice weekly following trypsinization. The supernatant, with titers of 10^4 - 10^5 /ml G-418 resistant colonies, was added to endothelial cells at two-thirds confluence and incubated for 12 hours in DMEM with 10% calf serum at 37° C. in 5% CO₂ in the presence of 8 μ g/ml of polybrene. Viral supernatants were removed, and cells maintained in medium 199 with 10% fetal calf serum, ECGS (50 μ g/ml), and endothelial cell conditioned medium (20%)

for an additional 24 to 48 hours prior to selection in G-418 (0.7 µg/ml of a 50% racemic mixture). G-418 resistant cells were isolated and analyzed for β -galactosidase expression using a standard histochemical stain (9). Cells stably expressing the β -galactosidase enzyme were maintained in continuous culture for use as needed. Frozen aliquots were stored in liquid nitrogen.

D. Immunotherapy of Malignancy by In Vivo Gene Transfer.

A retroviral vector which the H-2K^S gene was prepared. CT26 cells were infected with this vector in vitro, selected for G418 resistance, and analyzed by fluorescence activated cell sorting (FACS). Transduced CT26 cells showed a higher mean fluorescence intensity than uninfected CT26 cells or CT26 infected with different retroviral vectors. When 10⁶ CT26 cells which express H-2K^S were injected subcutaneously into BALB/c mice (H-2^d) sensitized to this antigen, no tumors were observed over an 8-week period in contrast to the unmodified CT26 (H-2^d) tumor line which routinely formed tumors at this dose. The immune response to H-2K^S could therefore provide protection against CT26 cells bearing this antigen. When CT26 H-2K^S and CT26 were co-inoculated, however, tumor growth was observed, suggesting that H-2K^S conferred sensitivity only to modified cells.

To determine whether protective effects could be achieved by introduction of H-2K^S in growing CT26 tumors, the recombinant H-2K^S reporter or a β -galactosidase gene was introduced into tumors either with a DNA/liposome or a retroviral vector. Tumor capsules (0.5–1 cm diameter) were exposed surgically and multiple needle injections (2–10) delivered to the parenchyma. With β -galactosidase reporter plasmids, recombinant gene expression could be readily detected after intra-tumor injection of DNA/liposome or retroviral vectors.

In mice which received intra-tumor injections of the H-2K^S DNA/liposome complex or H-2K^S retroviral vector, the recombinant DNA was detected by PCR in the tumor and occasionally in other tissues. When found in the other organs, no evidence of inflammation or organ toxicity was detected pathologically. An immune response to the recombinant H-2K^S protein was evident in these animals, however. Lymphocytes derived from the H-2K^S, but not β -galactosidase transduced tumors, demonstrated a cytolytic response to H-2K^S whether delivered by retroviral vectors or liposomes. More importantly, lymphocytes derived from the H-2K^S, but not β -galactosidase transduced animals, recognized and lysed unmodified CT26 cells, indicating that this stimulation induced immune reactivity against genetically unmodified tumor cells.

To assess the protective effect of the immune response against H-2K^S, tumor growth in vivo was quantitated. When animals received no prior sensitization to H-2K^S, one of four tumors transduced with H-2K^S showed attenuation of tumor growth which was not complete. In contrast, no anti-tumor effect was seen in unmodified (n=4) or β -galactosidase transduced controls (n=4). Because these tumors were large at the time of initial injection and continued to grow as the primary immune response was generated, an attempt was made to optimize the anti-tumor response by pre-immunization of mice with irradiated CT26 H-2K^S tumor cells, and by earlier and/or more frequent injections of vector. Tumors were transduced on days 12 and 32 by intra-tumor injection of H-2K^S or β -galactosi-

dase DNA/liposome vectors. Treatment with the H-2K^S liposome complex improved survival and attenuated tumor growth, in contrast to β -galactosidase transduced tumors where there was no difference in growth rate compared to the uninjected controls. Complete tumor regression was achieved in two mice by increasing the number of injections and by delivery of H-2K^S into tumors at an earlier stage. This treatment was protective, since control animals showed continued tumor growth and did not survive beyond 35 days.

E. Modulation of the Immune System.

The response to injection of cationic lipids and plasmids was determined after injection intravenously into BALB/c mice (6–12 weeks). In the first experiments, a gene encoding the H-2K^S molecule was introduced by tail vein injection. Two to four weeks later, spleen cells were harvested and analyzed for their ability to mediate a cytolytic T cell response. When these cells were tested using ⁵¹Cr target cells (CT26 cells expressing the H-2K^S gene), significant cytotoxicity was observed which was not seen in animals injected with the control vector, β -galactosidase (see FIG. 3). Up to 25% of target cells were lysed at effector: target ratios of 25:1.

In addition to this specific cytolytic T cell response, serologic or antibody responses to genes encoded by expression vector plasmids have been examined. When a plasmid encoding the gp160 molecule of HIV is injected, an antibody response is elicited in treated mice. In contrast to control animals injected with cationic lipids containing β -galactosidase, mice injected with cationic lipids with gp 160 plasmid showed an antibody response to the gp160 and gp120 form of this molecule by Western blot analysis (See FIG. 4). These results demonstrate that systemic administration of cationic lipid/DNA complexes can be used successfully to induce cell-mediated and antibody-mediated immunity against foreign pathogens.

F. Determination of Optimal Transfection Conditions.

(1) Plasmid Construction

A plasmid containing the *E. coli* lacZ gene under the control of the Rous Sarcoma Virus LTS (RSV- β -gal) (Norton and Coffin, *Mol. Cell. Biol.*, 5(2), 281–290, 1985) was used for transfection of porcine primary endothelial and HeLa cells. In addition, a plasmid containing the lacZ gene under the control of preproendothelin-1 5'-flanking DNA (–1410 to +83) (Wilson et al., *Mol. Cell. Biol.*, 10(9), 4854–4862, 1990) was used for transfection of endothelial cells. For in vivo toxicity analysis, the RSV- β -gal plasmid, and a plasmid derived from the PLJ vector containing the cDNA encoding an H-2K^S mouse MHC class I gene were used.

(2) Cell Culture, Transfection Analysis, and Toxicity in Vitro

Primary endothelial cells, derived from the Yucatan minipig (YPE cells), were incubated with medium 199 (M199) supplemented with 10% FBS, 2 mM l-glutamine, 50 U/ml penicillin, and 5 µg/ml streptomycin. HeLa cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 5% FBS, 2 mM l-glutamine, 50 U/ml penicillin and 5 µg/ml streptomycin. The DNA liposome mixture was prepared with lipid concentrations of DOPE/DC-Chol between 2.5 and 25 µM added to 0.2 ml of serum-free media or Ringer's lactate solution in polystyrene tubes. After mixing gently, the solution was allowed to stand at room temperature for 15–20 minutes. For transfection analysis, cells were grown in 60 mm tissue culture

dishes at 75% confluency or greater. Cells were washed twice with serum-free media or lactated Ringers solution and then placed in 0.5 mls of the same media. The DNA liposome solution (0.2 ml) was then added slowly to the cells, with gentle mixing, with a final volume of 0.7 ml. This resulted in DNA concentrations between 0.7 and 7 $\mu\text{g/ml}$ (13–130 nM), and lipid concentrations of 7–70 μM . Transfection was allowed to proceed for 1–5 hours, after which the cells were placed in media supplemented as described above. At 24–48 hours after transfection the enzymatic activity of the *E. coli* β -galactosidase protein was used to identify transfected cells by staining with the X-gal chromagen. Toxicity in vitro was assessed by cytopathic effect or trypan blue exclusion.

(3) Animal Studies

For intravenous injections, the DNA/liposomes were prepared as described for the in vitro transfection studies in 0.2 ml of serum-free M199 or lactated-Ringers solution. After 15–20 min of incubation, the mixture was diluted to 0.7 ml and 0.1 to 0.2 ml of this dilution was then injected immediately into the tail vein of adult, female BALB/c mice. Blood was collected before injection and 9–11 days following injection, and serum chemistries were examined. At ~2–3 weeks following injection, the liver, kidney, lung, heart, and brain were extracted for histologic and PCR DNA amplification analysis as described previously. Intratumor injection of CT26 cells (Fearon et al., *Cell*, 60, 397–403, 1990) and analysis were also performed according to the previous protocols.

(4) Results

The optimal conditions for transfection and toxicity of DNA/liposomes were initially determined in vitro. To obtain maximal transfection without toxicity in vitro, the ratio of DNA to cationic lipid, the absolute concentration of DNA or lipids, and the conditions for mixture of DNA and cationic lipids were studied. The cationic lipid preparation was a formulation of two compounds, which include dioleoyl phosphatidylethanolamine (DOPE) and cholesten-3- β -ol 3-urethanyl-N',N' dimethylethylene diamine (DC-chol). The transfection efficiencies of this reagent were equal to or greater than those of Lipofectin® (BRL) in several cell lines in vitro. Endothelial cells, which are typically difficult to transfect, and HeLa cells, which can be transfected easily using a variety of techniques, were examined by transfection in vitro.

To determine the optimal conditions for transfection of endothelial cells, the lipid was initially used at different concentrations while the DNA concentration was held constant. Maximal transfection efficiency was seen using 0.7 $\mu\text{g/ml}$ DNA (13 nM) and 21 μM of DOPE/DCChol lipid, with a sharp decline in the number of transfected cells with higher or lower lipid concentrations. Next, the DNA concentration was altered as the lipid concentration remained constant. This analysis revealed a similar sensitivity to DNA concentration, with the number of transfected cells decreasing significantly with increments of DNA concentration as low as 0.4 $\mu\text{g/ml}$. These results indicate that the ratio of DNA to lipid is important for maximum transfection efficiency, and that the absolute concentration of each component is also important in determining the efficiency of transfection. An increase in DNA and lipid concentration beyond the optimal concentration of 0.7 $\mu\text{g/ml}$ DNA (13 nM) and 21 μM of DOPE/DC-Chol reduced the number of viable cells and did not increase

the transfection efficiency of the remaining viable cells. Lipid concentrations greater than 35 μM reduced the number of viable cells by 50% compared to the untransfected control, whereas the optimal concentration of 0.7 $\mu\text{g/ml}$ DNA (13 nM) and 21 μM of lipid had no effect on cell viability after 5 hours of incubation.

To compare the optimal concentrations of transfection in a different cell type, transfections were performed on HeLa cells. In this case, a slightly different optimal ratio of DNA and lipid were observed. Peak transfection efficiencies were obtained at the same lipid concentration as endothelial cells (21 $\mu\text{g/ml}$) but varied less with small differences in DNA concentrations. DNA concentrations of 1.4–4.2 $\mu\text{g/ml}$ were equally effective. Again, when the ratio of DNA to lipid was maintained but the concentration of each was decreased three-fold, very few cells were transfected, illustrating that both the ratio of DNA to lipid and the absolute concentration of each component are important in maximizing the number of transfected cells. If HeLa cells were transfected at >80% confluence or greater, there was no toxicity using up to 35 μM of lipid. When cells were transfected at a lower saturation density, however, cell viability was reduced dramatically with as little as 7 μM of lipid compared to the untransfected control cells. These results demonstrate that the optimal conditions for transfection and toxicity may differ somewhat depending on the cell line.

Another variable in the preparation of liposomes was the composition of the solution used to generate complexes of the cationic lipids with DNA. Among several media solutions analyzed, no substantial difference was noted in transfection efficiency or toxicity with M199, McCoy's, OptiMEM, or RPMI media. A significant improvement in transfection efficiency was observed, however, using standard Ringers lactate. The number of transfected cells increased more than 3-fold compared to the serum-free medium, although prolonged incubation (≥ 2 hours) resulted in a loss of cell viability in some cell types.

PUBLICATIONS CITED

1. J. S. Reitman, R. W. Mahley, D. L. Fry, *Atherosclerosis* 43, 119 (1982).
2. R. E. Pitos, T. L. Innerarity, J. N. Weinstein, R. W. Mahley, *Arteriosclerosis* 1, 177 (1981); T. J. C. Van Berckel, J. F. Kruijt *FEBS Lett.* 132, 61 (1981); J. C. Voyta, P. A. Netland, D. P. Via, E. P. Zetter, *J. Cell. Biol.* 99, 81A (abstr.) (1984); J. M. Wilson, D. E. Johnston, D. M. Jefferson, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 4421 (1988).
3. J. Price, D. Turner, C. Cepko, *Proc. Natl. Acad. Sci. U.S.A.*, 84, 156 (1987).
4. R. Mann, R. C. Mulligan, D. Baltimore, *Cell* 33, 153 (1983); C. L. Cepko, B. E. Floberts, R. C. Mulligan, *Cell* 37, 1053 (1984); M. A. Eglitis, W. F. Anderson, *Biotechniques* 6, 608 (1988).
5. S. G. Ellis, G. S. Roubin, S. B. King, J. S. Douglas, W. S. Weintraub et al., *Circulation* 77, 372 (1988); L. Schwartz, M. G. Bourassa, J. Lesperance, H. E. Aldrige, F. Kazim, et al., *N. Engl. J. Med.* 318, 1714 (1988).
6. P. C. Block, R. K. Myler, S. Stertz, J. T. Fallon, *N. Engl. J. Med.* 305, 382 (1981); P. M. Steele, J. H. Chesebro, A. W. Stanson, *Circ. Res.* 57, 105 (1985); J. R. Wilentz, T. A. Sanborn, C. C. Handenschild, C. R. Valeri, T. J. Ryan, D. P. Faxon, *Circulation* 75, 636,

(1987); W. McBride, R. A. Lange, L. D. Hillis, *N. Engl. J. Med.* 318, 1734 (1988).

7. J. Folkman, M. Klagsbrun, *Science* 235, 442 (1987); S. J. Leibovich, P. J. Polverini, H. Michael Shepard, D. M. Wiseman, V. Shively, N. Nuseir, *Nature* 329, 630 (1987); J. Folkman, M. Klagsbrun, *Nature* 329, 671 (1987).

8. T. Matsumura, T. Yamanka, S. Hashizume, Y. Irie, K. Nitta, *Japan. J. Exp. Med.* 45, 377 (1975); D. G. S. Thilo, S. Muller-Kusel, D. Heinrich, I. Kauffer, E. Weiss, *Artery*, 8, 25a (1980).

9. A. M. Dannenberg, M. Suga, in *Methods for Studying Mononuclear Phagocytes*, D. O. Adams, P. J. Edelson, H. S. Koren, Eds. (Academic Press, New York, 1981), pp 375-395.

10. R. D. Cone, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* 81, 6349 (1984).

U.S. patent application Ser. Nos. 07/724,509, filed on Jun. 28, 1991, now pending, and 07/331,366, filed on Mar. 31, 1989, now abandoned, are incorporated herein by reference.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

We claim:

1. A kit for treating a disease in a patient in need thereof, comprising a catheter and a physiologically acceptable solution, wherein:

(i) said catheter is adapted for insertion into a blood vessel and comprises a main catheter body having a balloon element, adapted to be inserted in said blood vessel and being expansible against the walls of said vessel so as to hold said main catheter body in place, and means carried by said main catheter body for delivering said solution into said blood vessel;

(ii) said physiologically acceptable solution comprises DNA and at least one member selected from the group consisting of heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, and bivalent antibodies.

2. The kit of claim 1, wherein said physiologically acceptable solution further comprises a growth factor.

3. A kit for treating a disease in a patient in need thereof, comprising

(i) a catheter adapted for insertion into a blood vessel, comprising a main catheter body having a balloon element adapted to being inserted into said vessel and expansible against the walls of the said vessels so as to hold said main catheter body in place in said vessel and a means carried by said main catheter body for delivering a physiologically acceptable solution into said blood vessel;

(ii) said physiologically acceptable solution which may contain an enzyme, mild detergent or lipid; and

(iii) a means for causing a cell attached onto the walls of a vessel or in an organ or tissue in said patient to express an exogenous therapeutic agent protein, comprising a formulation adapted for delivery by said catheter for the transfer and uptake of RNA or DNA into said cell attached onto the walls of a vessel or in an organ or tissue in said patient.

4. The kit according to claim 3, wherein said DNA is antisense DNA.

5. The kit of claim 3, wherein said solution contains, as said enzyme, at least one member selected from the group consisting of dispase, trypsin, collagenase, papain, pepsin, chymotrypsin, and lipases.

6. The kit of claim 3, wherein said solution contains at least one member selected from the group consisting of Nonidet P-40, Triton X100, deoxycholate, and sodium dodecyl sulfate.

7. The kit of claim 3, wherein said main catheter body comprises two spaced balloon elements, adapted to be inserted in a blood vessel and both being expansible against the walls of the blood vessel, for providing a chamber in said blood vessel and so as to hold said main catheter body in place, and whereas said means for delivering a physiologically acceptable solution into said chamber is situated in between said balloon elements.

8. The kit of claim 3, wherein said means for delivering said solution into said blood vessel comprises a plurality of pore means.

9. The kit of claim 3, wherein said formulation comprises a retrovirus, a plasmid, a liposomal formulation, or a plasmid complex with a polycationic substance.

10. The kit of claim 3, wherein said formulation is a liposomal formulation.

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Subj: **New Patent application in the USA based on PCT/EP2007/053525**
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EVIDENCE APPENDIX

ITEM NO. 17

**Kornowski U.S. Patent No. 7,097,832
cited by Appellant as Exhibit B in the Response filed November 28, 2007**



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(12) **United States Patent**
Kornowski et al.

(10) **Patent No.:** **US 7,097,832 B1**
(45) **Date of Patent:** **Aug. 29, 2006**

(54) **INTRAMYOCARDIAL INJECTION OF
AUTOLOGOUS BONE MARROW**

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(21) Appl. No.: **09/868,411**

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§ 371 (c)(1),
(2), (4) Date: **Jun. 14, 2001**

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(51) **Int. Cl.**
A01N 63/00 (2006.01)
C12N 5/00 (2006.01)
C12N 5/06 (2006.01)

(52) **U.S. Cl.** **424/93.7; 435/384; 435/372**

(58) **Field of Classification Search** **424/94.1,**
424/577; 514/2, 21; 435/325

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,610,056 A * 3/1997 Nakahata 435/378
5,817,773 A 10/1998 Wilson et al.
5,980,893 A * 11/1999 Avraham et al. 424/144.1
5,997,860 A * 12/1999 Bauer et al. 424/93.71
6,013,780 A 1/2000 Neufeld et al.
6,676,937 B1 * 1/2004 Isner et al. 424/93.7
2005/0031600 A1 * 2/2005 Mickle et al. 424/93.7

FOREIGN PATENT DOCUMENTS

WO WO 91/04274 4/1991
WO WO 99/00486 1/1999
WO WO00/57922 10/2000

OTHER PUBLICATIONS

Perin et al. *Circulation*, 2003; 107 :2294-2302.*
Stamm et al. *Lancet*, 2003 ; 361 :45-6.*
Tao and Ma. *Pathology*, 2003; 35:6-13.*
Tomita et al. *Circulation*, 1999 ; 100(Supp II) :II-247-II-256.*

Chiu, RC. *Expert. Opin. Biol. Ther.* 2003; 3(2):215-25.*

Liechty et al. *Nat. Med.* 2000; 6(11): 1282-1286.*

Aoki et al. *Blood*. Jun. 1999; 93:4034-43.*

Mack et al. *J. Thor. Cardio. Surg.* 1998; 115:168-77.*

Cashman et al. *Blood*, 1998; 92: 2338-44.*

Fuchs et al. *J. Am. Coll. Cardiol.* 2001; 37: 1726-32.*

S. Fuchs, et al., "Transendocardial Delivery of Autologous Bone
Marrow Enhances Collateral Perfusion and Regional Function in
Pigs with Chronic Experimental Myocardial Ischemia," *Journal of
American College of Cardiology*, 2001, vol. 37, No. 6, pp. 1726-
1732.

C. Kalka, et al., "Angiogenesis and Vasculogenesis," *Herz*, 2000,
vol. 25, No. 6, pp. 611-622.

B.E. Strauer, M.D., et al., "Stem Cell Therapy in Perspective,"
Circulation, 2003, vol. 107, pp. 929-934.

Blume and Thomas, "A Review of Autologous Hematopoietic Cell
Transplantation," *Biology of Blood and Marrow Transplantation*,
vol. 6, 2000, pp. 1-12.

Brugger, Wolfram et al., "Ex Vivo Manipulation of Hematopoietic
Stem and Progenitor Cells," *Seminars in Hematology*, vol. 37, No.
1, Supplement 2, Jan. 2000, pp. 42-49.

Dini, G. et al., "Peripheral Blood Stem Cell Collection from
G-CSF-Stimulated Unrelated Donors for Second Transplant," *Bone
Marrow Transplantation*, vol. 22, Supplement 5, 1998, pp. S41-S45.

Fielding and Goldstone, "Autologous Bone Marrow Transplanta-
tion," *Current Opinion in Hematology*, vol. 1, 1994, pp. 412-417.

Kanj, Souha S. et al., "Myocardial Ischemia Associated With
High-Dose Carmustine Infusion," *Cancer*, vol. 68, Nov. 1, 1991, pp.
1910-1912.

Noishiki, Yasuharu et al., "Angiogenic Growth Factor Release
System for In Vivo Tissue Engineering: A Trial of Bone Marrow
Transplantation into Ischemic Myocardium," *J. Artif. Organs*, vol.
2, 1999, pp. 85-91.

Sinnett, S.J. et al., "Autologous Stem Cell Transplantation for
Malignancy: A Systematic Review of the Literature," *Clin. Lab.
Haem.*, vol. 22, 2000, pp. 61-72.

Vaughan, William P. et al., "Roundtable Discussion: Incorporating
Bone Marrow Transplantation Into NCCN Guidelines," *Oncoology*,
vol. 12, No. 11A, NCCN Proceedings, Nov. 1998, pp. 390-392.

(Continued)

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(57) **ABSTRACT**

A method of treating cardiac or myocardial conditions
comprises the administration of an effective amount of
autologous bone marrow. The bone marrow may optionally
be stimulated and/or administered in combination with a
pharmaceutical drug, protein, gene or other factor or therapy
that may enhance bone marrow production of angiogenic
growth factors and/or promote endothelial cell proliferation
or migration or blood vessel formation.

15 Claims, 2 Drawing Sheets

OTHER PUBLICATIONS

- Dubrey et al., "Improvement of Echocardiographic and Electrocardiographic features in AL Cardiac Amyloidosis with Intensive Melphalan and Autologous Stem Cell Rescue" *Boston University School of Medicine*, XP-00107 135.
- Isner and Asahara, "Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization" *The Journal of Clinical Investigation* 103 (9):1231-1236 (1999).
- Karen K. Fields, "Autologous Bone Marrow Transplantation and Melanoma: A Focused Review of the Literature" *Annals of Plastic Surgery* 28 (1):70-73 (1992), XP-001069809.
- Noishiki et al., "Autocrine angiogenic vascular prosthesis with bone marrow transplantation" *Nature Medicine*, 2 (1):90-93 (1996).
- Tomita et al., "Autotransplanted Mesenchymal Stem Cells Improve Functions After A Myocardial Infarction," *Circulation* 98 (17):1200 Abstract 1037.
- Yamauchi et al., "New agents for the treatment of infarcted myocardium" *The FASEB Journal* 10.1096/fj.99-0565fje (2000).
- S. Fuchs, et al., "Transendocardial Delivery of Autologous Bone Marrow Enhances Collateral Perfusion and Regional Function in Pigs with Chronic Experimental Myocardial Ischemia," *Journal of American College of Cardiology*, 2001, vol. 37, No. 6, pp. 1726-1732.
- C. Kalka, et al., "Angiogenesis and Vasculogenesis," *Herz*, 2000, vol. 25, No. 6, pp. 611-622.
- B.E. Strauer, M.D., et al., "Stem Cell Therapy in Perspective," *Circulation*, 2003, vol. 107, pp. 929-934.
- Anderson, "Human gene therapy", *Nature*, 392(6679 Supp.):25-30 (Apr. 1998).
- Bickenbach et al., "Selection and Extended Growth of Murine Epidermal Stem Cells in Culture", *Exp. Cell Res.*, 244(1):184-195 (Oct. 1998).
- Blanquaert et al., "RGTA modulates the healing pattern of a defect in a monolayer of osteoblastic cells by acting on both proliferation and migration", *J. Biomed. Mater. Res.*, 64A(3):525-532 (Mar. 2003).
- Asahara, T. et al., "Gene therapy of endothelial progenitor cell for vascular development in severe ischemic disease", *Circulation*, vol. 100, (1999) pp. 1-481, Abstract XP009051610.
- Isner, Jeffrey M., "Myocardial gene Therapy", *Nature* (London) vol. 415, No. 6868, (2002) pp. 234-239.
- Chiu, "Adult stem cell therapy for heart failure". *Expert Opin Biol Ther.*, Apr. 2003, 3(2):215-25.
- Desgranges et al., "Beneficial use of fibroblast growth factor 2 and RGTA, a new family of heparan mimics, for endothelialization of PET prostheses", *J Biomed Mater Res.*, 2001, 53(1):1-9.
- Dowell et al., "Myocyte and myogenic stem cell transplantation in the heart", *Cardiovasc Res.*, May 2003, 58(2):336-50.
- Folkman, "Therapeutic angiogenesis in ischemic limbs", *Circulation*, Mar. 1998, 97(12):1108-10.
- Iwaguro et al., "Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration", *Circulation*, Feb. 2002, 105(6):732-8.

* cited by examiner

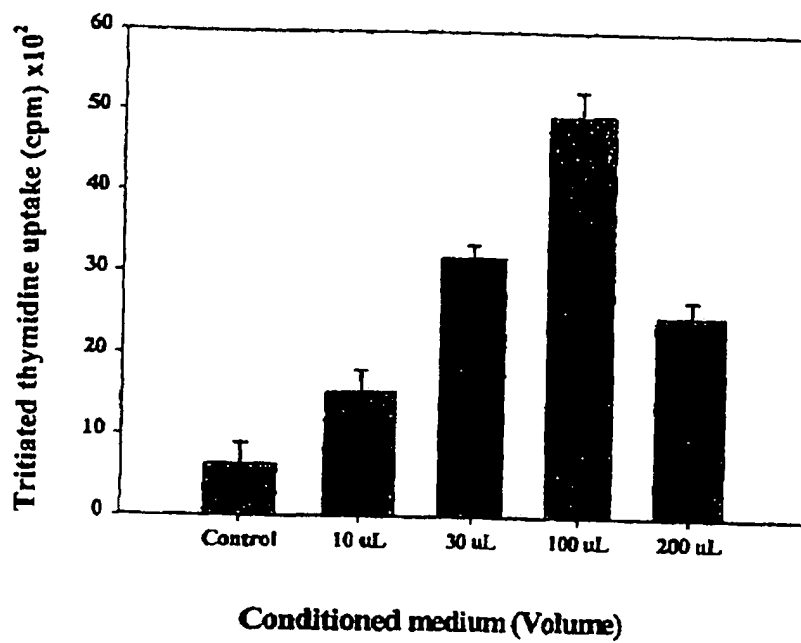


Fig. 1

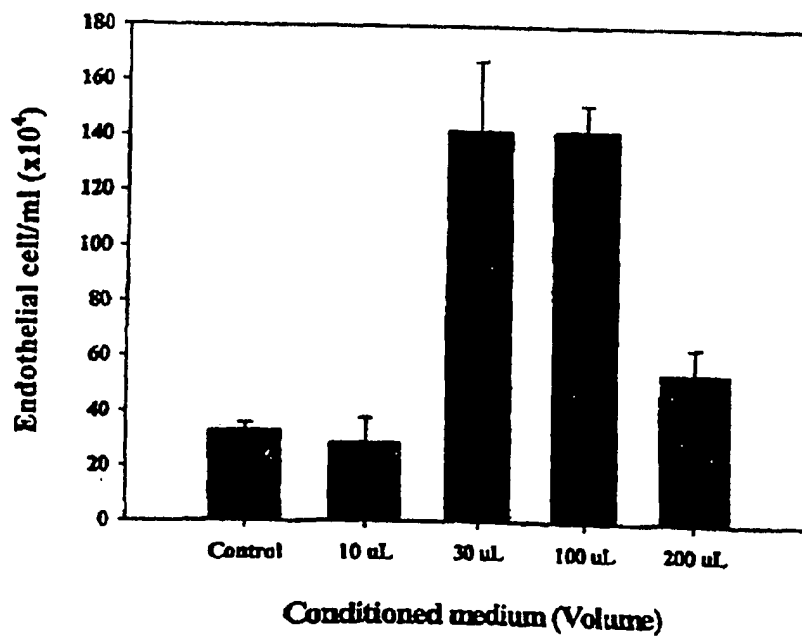


Fig. 2

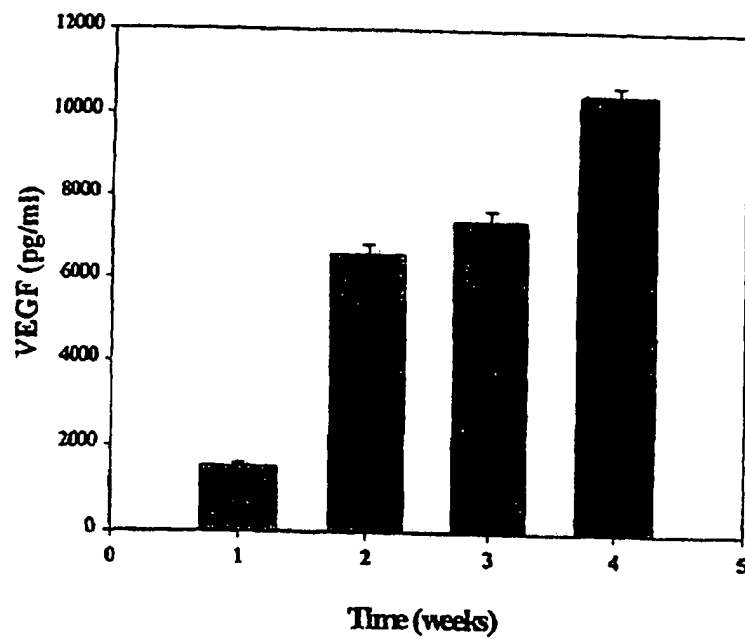


Fig. 3

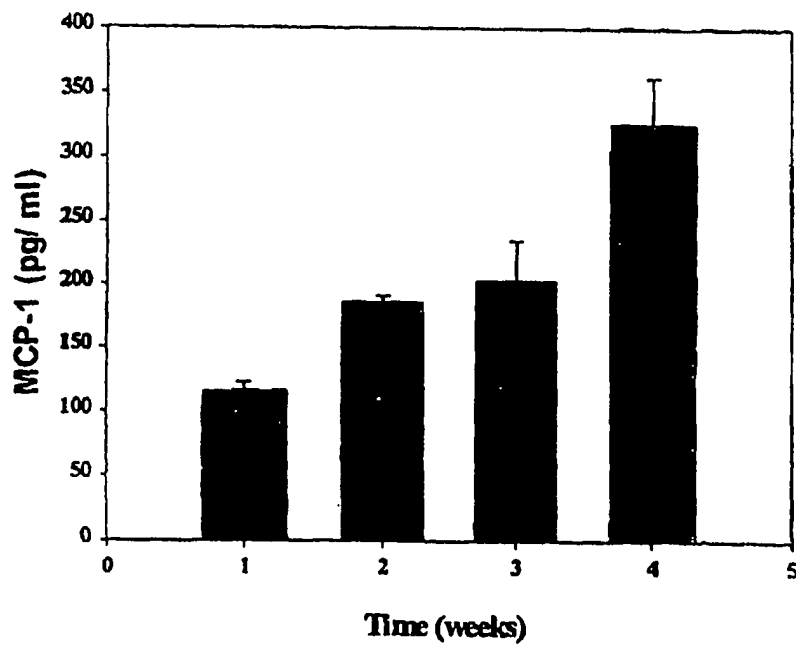


Fig. 4

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INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage application of international application PCT/US00/08353, filed 30 Mar. 2000, which claims benefit to U.S. Provisional Application Nos. 60/126,800, filed 30 Mar. 1999, and 60/138,379, filed 9 Jun. 1999.

FIELD OF THE INVENTION

This application is directed to a method of injecting autologous bone marrow. More specifically, this invention is directed to intramyocardial injection of autologous bone marrow to enhance collateral blood vessel formation and tissue perfusion.

BACKGROUND OF THE INVENTION

The use of recombinant genes or growth-factors to enhance myocardial collateral blood vessel function may represent a new approach to the treatment of cardiovascular disease. Komowski, R., et al., "Delivery strategies for therapeutic myocardial angiogenesis", *Circulation* 2000; 101:454-458. Proof of concept has been demonstrated in animal models of myocardial ischemia, and clinical trials are underway. Unger, E. F., et al., "Basic fibroblast growth factor enhances myocardial collateral flow in a canine model", *Am J Physiol* 1994; 266:H1588-1595; Banai, S. et al., "Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs", *Circulation* 1994; 83:2189; Lazarous, D. F., et al., "Effect of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart", *Circulation* 1995; 91:145-153; Lazarous, D. F., et al., "Comparative effects of basic development and the arterial response to injury", *Circulation* 1996; 94:1074-1082; Giordano, F. J., et al., "Intracoronary gene transfer of fibroblast growth factor-S increases blood flow and contractile function in an ischemic region of the heart", *Nature Med* 1996; 2:534-9. Most strategies for trans-catheter delivery of angiogenic factors have employed an intracoronary route which may have limitations due to imprecise localization of genes or proteins and systemic delivery to non-cardiac tissue. Thus, it would be desirable to have the capacity for direct delivery of angiogenic factors or genes to precisely defined regions of the myocardium rather than to the entire heart, and to minimize the potential for systemic exposure. Guzman, R. J., et al., "Efficient gene transfer into myocardium by direct injection of adenovirus vectors", *Circ Res* 1993; 73:1202-7; Mack, C. A., et al., "Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for VEGF-121, improves myocardial perfusion and function in the ischemic porcine heart", *J Thorac Cardiovasc Surg* 1998; 115:168-77.

The effect of direct intra-operative intramyocardial injection of angiogenic factors on collateral function has been studied in animal models of myocardial ischemia. Open chest, transepical administration of an adenoviral vector containing a transgene encoding an angiogenic peptide resulted in enhanced collateral function. (Mack et al., supra.) Angiogenesis was also reported to occur with direct intramyocardial injection of an angiogenic peptide or a

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plasmid vector during open heart surgery in patients. Schumacher, B., et al., "Induction of neoangiogenesis in ischemic myocardium by human growth factors. First clinical results of a new treatment of coronary heart disease", *Circulation* 1998; 97:645-650; Losordo, D. W., et al., "Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia", *Circulation* 1998; 98:2800.

Despite the promising hope for therapeutic angiogenesis as a new modality to treat patients with coronary artery disease, there is still a huge gap regarding what specific strategy will optimally promote a clinically relevant therapeutic angiogenic response. Moreover, it is unclear which one (or more) out of multiple angiogenic growth factors may be associated with a beneficial angiogenic response. In addition, the use of different tissue delivery platforms, e.g., proteins, adenovirus, or "naked" DNA, to promote the optimal angiogenic response has remained an open issue.

OBJECTS OF THE INVENTION

It is an object of this invention to provide a novel therapeutic modality wherein autologous bone marrow is injected to promote angiogenesis in the injected tissue.

It is also an object of this invention to provide a novel method of intramyocardial injection to enhance collateral blood vessel formation and tissue perfusion.

These and other objects of the invention will become more apparent in the discussion below.

SUMMARY OF THE INVENTION

Most currently tested therapeutic approaches have focused on a single angiogenic growth factor (e.g., VEGF, FGF, angiopoietin-1) delivered to the ischemic tissue. This can be accomplished either by delivery of the end-product (e.g., protein) or by gene transfer, using diverse vectors. However, it is believed that complex interactions among several growth factor systems are probably necessary for the initiation and maintenance of new blood vessel formation. More specifically, it is believed important to induce a specific localized angiogenic milieu with various angiogenic cytokines interacting in concert and in a time-appropriate manner to initiate and maintain the formation and function of new blood vessels.

The bone marrow (BM) is a natural source of a broad spectrum of cytokines and cells that are involved in the control of angiogenic processes. It is therefore believed that the intramyocardial injection of autologous (A) BM, by taking advantage of the natural ability of these cells to secrete many angiogenic factors in a time-appropriate manner, provides an optimal intervention for achieving therapeutic collateral development in ischemic myocardium.

According to the invention autologous bone marrow is injected, either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Non-limiting examples of these "combined" agents are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP-1), and Hypoxia Inducible Factor-1 (HIF-1). An example of an intervention that may enhance bone

marrow production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. The autologous bone marrow, alone or with "combined" agents, can be delivered to the patient directly via either trans-endocardial or trans-epicardial approaches into either ischemic and/or non-ischemic myocardium, or directly into any other ischemic organ (including a peripheral limb) to enhance and/or promote the development of collateral blood vessel formation and therefore collateral flow to ischemic myocardium or ischemic limbs. This approach can also be used to promote the development of newly implanted dedifferentiated and/or differentiated myocardial cells by the process of cardiac myogenesis.

The invention comprises various autologous bone marrow transplantation strategies to enhance angiogenesis and/or myogenesis and thereby accelerate the development of new blood vessels into ischemic myocardium or lower extremities. Another aspect of the invention concerns the strategy of "optimization of angiogenic gene expression." This strategy employs co-administration of HIF-1 with the autologous bone marrow. HIF-1 is a transcription factor known to be induced and activated by hypoxia, and known to induce expression of multiple genes involved in the response to hypoxia. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1. The strategy also involves the ex-vivo exposure of the bone marrow to hypoxia to increase the production of vascular endothelial growth factor (VEGF) expression or other cytokines with proven angiogenic activity (such as MCP-1) prior to its direct injection into the heart or any peripheral ischemic tissue. This invention thus includes the direct intramyocardial (trans-epicardial or trans-endocardial) or peripheral intramuscular injection of autologous bone marrow; stimulated autologous bone marrow, for example, stimulated by HIF-1, EPAS1, MCP-1, GM-CSF, or transient exposure to hypoxia or other forms of energy, such as ultrasound, RF, electromagnetic or laser energy; or autologous bone marrow product derived from conditioned medium (acellular component/s of cultured bone marrow). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the proliferation of PAEC's vs. the quantities of conditioned medium;

FIG. 2 is a graph of the proliferation of endothelial cells vs. the quantities of conditioned medium;

FIG. 3 is a graph of the concentration of VEGF in conditioned medium over a four-week period of time; and

FIG. 4 is a graph of the concentration of MCP-1 in conditioned medium over a four-week period of time.

DETAILED DESCRIPTION OF THE INVENTION

Bone marrow is a natural source of a broad spectrum of cytokines that are involved in the control of angiogenic and inflammatory processes. The cytokines expressed comprise mediators known to be involved in the maintenance of early and late hematopoiesis (IL-1 alpha and IL-1 beta, IL-6, IL-7,

IL-8, IL-11 and IL-13; colony-stimulating factors, thrombopoietin, erythropoietin, stem cell factor, fit 3-ligand, hepatocyte cell growth factor, tumor necrosis factor alpha, leukemia inhibitory factor, transforming growth factors beta 1 and beta 3; and macrophage inflammatory protein 1 alpha), angiogenic factors (fibroblast growth factors 1 and 2, vascular endothelial growth factor) and mediators whose usual target (and source) is the connective tissue-forming cells (platelet-derived growth factor A, epidermal growth factor, transforming growth factors alpha and beta 2, oncostatin M and insulin-like growth factor-1), or neuronal cells (nerve growth factor). Sensebe, L., et al., *Stem Cells* 1997; 15:133-43. Moreover, it has been shown that VEGF polypeptides are present in platelets and megacaryocytes, and are released from activated platelets together with the release of beta-thromboglobulin. Wartiovaara, U., et al., *Thromb Haemost* 1998; 80:171-5; Mohle, R., *Proc Natl Acad Sci USA* 1997; 94:663-8.

There are also indicators to support the concept that angiogenesis is needed to support bone marrow function and development of hematopoietic cells, including stem cells and progenitor cells, that may enter the circulation and target to sites of wound healing and/or ischemia, ultimately contributing to new blood vessel formation. Monoclonal antibodies that specifically recognize undifferentiated mesenchymal progenitor cells isolated from adult human bone marrow have been shown to recognize cell surface markers of developing microvasculature, and evidence suggests such cells may play a role in embryonal angiogenesis. Fleming, J. E., Jr., *Dev Dyn* 1998; 212:119-32.

Bone marrow angiogenesis may become exaggerated in pathologic states where the bone marrow is being activated by malignant cells (such as in multiple myeloma) where bone marrow angiogenesis has been shown to increase simultaneously with progression of human multiple myeloma cells. Ribatti, D., et al., *Br J Cancer* 1999; 79:451-5. Moreover, vascular endothelial growth factor (VEGF) has been shown to play a role in the growth of hematopoietic neoplasms such as multiple myeloma, through either a paracrine or an autocrine mechanism. Bellamy, W. T., *Cancer Res* 1999; 59:728-33; Fiedler, W., *Blood* 1997; 89:1870-5. It is believed that autologous bone marrow, with its unique native humoral and cellular properties, is a potential source of various angiogenic compounds. This natural source of "mixed" angiogenic cytokines can surprisingly be utilized as a mixture of potent interactive growth factors to produce therapeutic angiogenesis and/or myogenesis; use of the cells per se could provide a more sustained source of these natural angiogenic agents.

One of the factors that most likely participates in initiating angiogenesis in response to ischemia is HIF-1, a potent transcription factor that binds to and stimulates the promoter of several genes involved in responses to hypoxia. Induction and activation of HIF-1 is tightly controlled by tissue pO₂; HIF-1 expression increases exponentially as pO₂ decreases, thereby providing a positive feedback loop by which a decrease in pO₂ causes an increase in the expression of gene products that serve as an adaptive response to a low oxygen environment. Activation of HIF-1 leads, for example, to the induction of erythropoietin, genes involved in glycolysis, and to the expression of VEGF. It probably also modulates the expression of many other genes that participate in the adaptive response to low pO₂ levels. The mechanism by which HIF-1 regulates levels of proteins involved in the response to hypoxia is through transcriptional regulation of genes responding to low pO₂. Thus, such genes have short DNA sequences within the promoter or enhancer regions

that contain HIF-1 binding sites, designated as hypoxia responsive elements (HRE). HIF-1 is a heterodimer with a basic helix-loop-helix motif, consisting of the subunits HIF-1 α and HIF-1 β . Its levels are regulated by pO₂ both transcriptionally and posttranscriptionally—HIF-1 induction is increased by hypoxia, and its half-life is markedly reduced as pO₂ levels increase.

It is relevant that while expression of HIF-1 (as determined in HeLa cells) is exponentially and inversely related to pO₂, the inflection point of the curve occurs at an oxygen saturation of 5%, with maximal activity at 0.5% and 1/2 maximal activity at 1.5–2.0%. These are relatively low levels of hypoxia, and it is not clear whether such levels occur in the presence of mild levels of myocardial or lower limb ischemia—i.e., levels present in the absence of tissue necrosis (myocardial infarction, and leg ulcerations, respectively). Thus, bone marrow cells could have the capacity to secrete angiogenic factors and thereby enhance collateral development. However, it is possible that such activity may not become manifest in the specific tissue environments treated unless some additional stimulus is present. It is, therefore, a preferred aspect of the invention to co-administer, if necessary, bone marrow implant with HIF-1. It is anticipated that HIF-1 will provide optimal expression of many of the hypoxia-inducible angiogenic genes present in the bone marrow implant. The HIF-1 can be injected either as the protein, or as the gene. If as the latter, it can be injected either in a plasmid or viral vector, or any other manner that leads to functionally relevant protein levels. For example, bone marrow can be transfected, ex vivo, with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. It is emphasized, however, that HIF-1 is used in this section as an example of an intervention that could enhance production of angiogenic substances by bone marrow. This invention also covers use of other agents, which by enhancing HIF-1 activity (i.e., prolonging its half-life), or by producing effects analogous to HIF-1, stimulate the bone marrow to increase expression of angiogenic factors. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1.

Because VEGF promoter activity is enhanced by HIF-1, this invention also includes the ex-vivo exposure of bone marrow cells in culture to hypoxia or other forms of energy, such as, for example, ultrasound, RF, or electromagnetic energy. This intervention increases VEGF and other gene expression. By this effect it may augment the capacity of bone marrow to stimulate angiogenesis.

Another aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by HIF-1 (or products that augment the effects of HIF-1 or produce similar effects to HIF-1 on bone marrow) or direct exposure of bone marrow to hypoxic environment followed by the delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant

genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

Current data indicate the importance of monocyte-derived cytokines for enhancing collateral function. Monocytes are activated during collateral growth in vivo, and monocyte chemotactic protein-1 (MCP-1) is upregulated by shear stress in vitro. It has been shown that monocytes adhere to the vascular wall during collateral vessel growth (arteriogenesis) and capillary sprouting (angiogenesis). MCP-1 was also shown to enhance collateral growth after femoral artery occlusion in the rabbit chronic hindlimb ischemia model (Ito et al., *Circ Res* 1997; 80:829–33). Activation of monocytes seems to play an important role in collateral growth as well as in capillary sprouting. Increased monocyte recruitment by LPS is associated with increased capillary density as well as enhanced collateral and peripheral conductance at 7 days after experimental arterial occlusion (Arras M. et al., *J Clin Invest* 1998;101:40–50.)

A further aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by MCP-1, followed by the direct delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion and muscular function in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to MCP-1 in the form of the protein, or the bone marrow cells can be transfected with a vector carrying the MCP-1 gene. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the MCP-1 transgene.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Granulocyte-Colony Stimulatory Factor (G-CSF) are stimulatory cytokines for monocyte maturation and are multipotent hematopoietic growth factors, which are utilized in clinical practice for various hematological pathologies such as depressed white blood cell count (i.e., leukopenia or granulocytopenia or monocytopenia) which occurs usually in response to immunosuppressive or chemotherapy treatment in cancer patients. GM-CSF has also been described as a multilineage growth factor that induces in vitro colony formation from erythroid burst-forming units, eosinophil colony-forming units (CSF), and multipotential (CSF), as well as from granulocyte-macrophage CSF and granulocyte CFU. (Bot F. J., *Exp Hematol* 1989, 17:292–5). Ex-vivo exposure to GM-CSF has been shown to induce rapid proliferation of CD-34+ progenitor cells (Egeland T. et al., *Blood* 1991; 78:3192–9.) These cells have the potential to differentiate into vascular endothelial cells and may naturally be involved in postnatal angiogenesis. In addition, GM-CSF carries multiple stimulatory effects on macrophage/monocyte proliferation, differentiation, motility and survival (reduced apoptotic rate). Consistent with the combined known effects on bone marrow derived endothelial progenitor cells and monocytes, it is another aspect of the invention to use GM-CSF as an adjunctive treatment to autologous bone marrow injections aimed to induce new blood vessel formation and differentiation in ischemic cardiovascular organs. Moreover, GM-CSF may further enhance therapeutic myocardial angiogenesis caused by bone marrow, by augmenting the effect of bone marrow, or by further stimulating, administered either in vivo or in vitro, bone marrow that is also being stimulated by agents such as HIF-1, EPAS1, hypoxia, or MCP-1.

In the examples below, certain testing regarding aspects of the invention is set forth. These examples are non-limitative.

EXAMPLES

Example 1

Effect of Bone Marrow Cultured Media on Endothelial Cell Proliferation

Studies were conducted to determine whether aspirated pig autologous bone marrow cells obtained secreted VEGF, a potent angiogenic factor, and MCP-1, which recently has been identified as an important angiogenic co-factor. Bone marrow was cultured *in vitro* for four weeks. The conditioned medium was added to cultured pig aortic endothelial cells (PAECs), and after four days proliferation was assessed. VEGF and MCP-1 levels in the conditioned medium were assayed using ELISA. During the four weeks in culture, BM cells secreted VEGF and MCP-1, such that their concentrations increased in a time-related manner. The resulting conditioned medium enhanced, in a dose-related manner, the proliferation of PAECs. The results indicate that BM cells are capable of secreting potent angiogenic cytokines such as VEGF and MCP-1 and of inducing proliferation of vascular endothelial cells.

Pig Bone Marrow Culture

Bone marrow (BM) cells were harvested under sterile conditions from pigs with chronic myocardial ischemia in preservative free heparin (20 units/ml BM cells) and filtered sequentially using 300 μ and 200 μ stainless steel mesh filters. BM cells were then isolated by Ficoll-Hypaque gradient centrifugation and cultured in long-term culture medium (LTCM) (Stem Cell Tech, Vancouver, British Columbia, Canada) at 33 $^{\circ}$ C. with 5% CO $_2$ in T-25 culture flask. The seeding density of the BMCs in each culture was 7 \times 10 6 /ml. Weekly, one half of the medium was removed and replaced with fresh LTCM. The removed medium was filtered (0.2 μ filter) and stored at -200 $^{\circ}$ C. for subsequent Enzyme-linked Immunosorbent Assay (ELISA) and cell proliferation assays.

Isolation and Culture of Pig Aortic Endothelial Cells

Fresh pig aortic endothelial cells (PAECs) were isolated using conventional methods. Endothelial cell growth medium (EGM-2 medium, Clonetics, San Diego, Calif.), containing 2% FBS, hydrocortisone, human FGF, VEGF, human EGF, IGF, heparin and antibiotics, at 37 $^{\circ}$ C. with 5% carbon dioxide. When the cells became confluent at about 7 days, they were split by 2.5% trypsin and cultured thereafter in medium 199 with 10% FBS. Their identity was confirmed by typical endothelial cell morphology and by immunohistochemistry staining for factor VIII. Passage 3-10 were used for the proliferation study.

Effects of Conditioned Medium on Aortic Endothelial Cells

Cell proliferation assay: PAECs (Passage 3-10) were removed from culture flasks by trypsinization. The detached cells were transferred to 96-well culture plates and plated at a seeding density of 5,000 cells/well. Cells were cultured for 2-3 days before being used in proliferation and DNA synthesis experiments. The conditioned medium of BM cells cultures were collected at 4 weeks; medium from 7 culture flasks were pooled and used in the bioassay. Aliquotes (10 μ L, 30 μ L, 100 μ L or 200 μ L) of pooled conditioned medium, or LTCM (200 μ L, as control), were added to confluent PAECs in 96-well plates in triplicate. Four days following culture with conditioned medium or control medium, the PAECs were trypsinized and counted using a cell counter (Coulter Counter Beckman Corporation, Miami Fla.).

Effects of Conditioned Medium on PAEC DNA Synthesis

Aliquotes (10 μ L, 30 μ L, 100 μ L or 200 μ L) of conditioned medium from pooled samples or control medium (LTCM, 200 μ L) were added to PAECs in 96-well plate (same seeding density as above) in triplicate. After 2 days, 1 μ Ci tritiated thymidine was added to each well. Forty-eight hours later, DNA in PAECs was harvested using a cell harvester (Mach III M Tomtec, Hamden, Conn.) and radioactivity was counted by liquid scintillation counter (Multi-detector Liquid Scintillation Luminescence Counter EG&G Wallac, Turku, Finland).

Determination of VEGF and MCP-1 in Conditioned Medium by ELISA VEGF

The concentration of VEGF in conditioned medium was measured using a sandwich ELISA kit (Chemicon International Inc., Temecula, Calif.). Briefly, a plate pre-coated with anti-human VEGF antibody was used to bind VEGF in the conditioned medium or to a known concentration of recombinant VEGF. The complex was detected by the biotinylated anti-VEGF antibody, which binds to the captured VEGF. The biotinylated VEGF antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human VEGF antibody cross-reacts with porcine VEGF.

Determination of MCP-1 in Conditioned Medium by ELISA

The concentration of MCP-1 in conditioned medium was assayed by sandwich enzyme immunoassay kit (R & D Systems, Minneapolis, Minn.): a plate pre-coated with anti human MCP-1 antibody was used to bind MCP-1 in the conditioned medium or to a known concentration of recombinant protein. The complex was detected by the biotinylated anti-MCP-1 antibody, which binds to the captured MCP-1. The biotinylated MCP-1 antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human MCP-1 antibody cross-reacts with porcine MCP-1.

Results

The BM conditioned medium collected at four weeks increased, in a dose-related manner, the proliferation of PAECs (FIG. 1). This was demonstrated by counting the number of cells directly and by measuring tritiated thymidine uptake ($p < 0.001$ for both measurements). The dose-related response demonstrated a descending limb; proliferation decreased with 200 μ L conditioned medium compared to 30 μ L and 100 μ L ($P = 0.003$ for both comparisons). Similar dose-related results were observed in the tritiated thymidine uptake studies ($P = 0.03$ for 30 μ L and 100 μ L compared to 200 μ L, respectively).

A limited number (5 \pm 4%) of freshly aspirated BM cells stained positive for factor VIII. The results are set forth in FIG. 2. This contrasted to 57 \pm 14% of the adherent layer of BM cells cultured for 4 weeks, of which 60 \pm 23% were endothelial-like cells and 40 \pm 28% appeared to be megakaryocytes.

Over a 4-week period, the concentrations of VEGF and MCP-1 in the BM conditioned medium increased gradually to 10 and 3 times the 1st week level, respectively ($P < 0.001$ for both comparisons) (FIG. 3). In comparison, VEGF and MCP-1 levels in a control culture medium, not exposed to BM, were 0 and 11 \pm 2 pg/ml, respectively, as shown in FIG.

Example 2

Effects of Hypoxia on VEGF Secretion by Cultured Pig Bone Marrow Cells

It was demonstrated that hypoxia markedly increases the expression of VEGF by cultured bone marrow endothelial cells, results indicating that ex-vivo exposure to hypoxia, by increasing expression of hypoxia-inducible angiogenic factors, can further increase the collateral enhancing effect of bone marrow cells and its conditioned media to be injected in ischemic muscular tissue. Pig bone marrow was harvested and filtered sequentially using 300 μ and 200 μ stainless steel mesh filters. BMCs were then isolated by Ficoll-Hypaque gradient centrifugation and cultured at 33° C. with 5% CO₂ in T-75 culture flasks. When cells became confluent at about 7 days, they were split 1:3 by trypsinization. After 4 wks of culture, the BMCs were either exposed to hypoxic conditions (placed in a chamber containing 1% oxygen) for 24 to 120 hrs, or maintained under normal conditions. The resulting conditioned medium was collected and VEGF, MCP-1 were analyzed by ELISA.

Exposure to hypoxia markedly increased VEGF secretion: At 24 hrs VEGF concentration increased from 106 \pm 13 pg/ml under normoxic, to 1,600 \pm 196 pg/ml under hypoxic conditions ($p=0.0002$); after 120 hrs it increased from 4,163 \pm 62 to 6,028 \pm 167 pg/ml ($p<0.0001$). A separate study was performed on freshly isolated BMCs, and the same trend was found. Hypoxia also slowed the rate of proliferation of BMCs. MCP-1 expression was not increased by hypoxia, a not unexpected finding as its promoter is not known to have HIF binding sites.

Example 3

Effect of Bone Marrow Cultured Media on Endothelial Cell Tube Formation

It was demonstrated, using pig endothelial cells and vascular smooth muscle cells co-culture technique, that the conditioned medium of bone marrow cells induced the formation of structural vascular tubes in vitro. No such effect on vascular tube formation was observed without exposure to bone marrow conditioned medium. The results suggest that bone marrow cells and their secreted factors exert pro-angiogenic effects.

Example 4

The effect of Transendocardial Delivery of Autologous Bone Marrow on Collateral Perfusion and Regional Function in Chronic Myocardial Ischemia Model

Chronic myocardial ischemia was created in 14 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. Four weeks after implantation, 7 animals underwent transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 7 control animals were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone \times 100)

improved in ABM-treated pigs but not in controls (ABM: 95 \pm 13 vs 81 \pm 11 at rest, $P=0.017$; 85 \pm 19 vs 72 \pm 10 during adenosine, $P=0.046$; Controls: 86 \pm 14 vs 86 \pm 14 at rest, $P=NS$; 73 \pm 17 vs 72 \pm 14 during adenosine, $P=0.63$). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 83 \pm 21 vs 60 \pm 32 at rest, $P=0.04$; 91 \pm 44 vs 35 \pm 43 during pacing, $P=0.056$, Controls: 69 \pm 48 vs 64 \pm 46 at rest, $P=0.74$; 65 \pm 56 vs 37 \pm 56 during pacing, $P=0.23$).

The results indicate that catheter-based transendocardial injection of ABM can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Fourteen specific-pathogen-free domestic pigs weighing approximately 70 kg were anesthetized, intubated, and received supplemental O₂ at 2 L/min as well as 1-2% isoflurane inhalation throughout the procedure. Arterial access was obtained via right femoral artery isolation and insertion of an 8 French sheath. The left circumflex artery was isolated through a left lateral thoracotomy and a metal encased ameroid constrictor was implanted at the very proximal part of the artery. Four weeks after the ameroid constrictor implantation all pigs underwent (1) a selective left and right coronary angiography for verification of ameroid occlusion and assessment of collateral flow; (2) transthoracic echocardiography studies; and (3) regional myocardial blood flow assessment.

Bone Marrow Aspiration and Preparation and Intramyocardial Injection

Immediately after completion of the baseline assessment, all animals underwent BM aspiration from the left femoral shaft using standard techniques. BM was aspirated from 2 sites (3 ml per site) using preservative free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow was immediately macro-filtered using 300 μ and 200 μ stainless steel filters, sequentially. Then, the bone marrow was injected using a trans-endocardial injection catheter into the myocardium in 12 sites (0.2 ml per injection site for total of 2.4. ml) directed to the ischemic myocardial territory and its borderline region.

Echocardiography Study

Transthoracic echocardiography images of short and long axis views at the mid-papillary muscle level were recorded in animals at baseline and during pacing, at baseline and during follow-up evaluation at four weeks after ABM implantation. Fractional shortening measurements were obtained by measuring the % wall thickening (end-systolic thickness minus end-diastolic thickness/end-diastolic thickness) \times 100. Those measurements were taken from the ischemic territory (lateral area) and remote territory (anterior-septal area). Subsequently, a temporary pacemaker electrode was inserted via a right femoral venous sheath and positioned in the right atrium. Animals were paced at 180/minute for 2 minutes and echocardiographic images were simultaneously recorded.

Regional Myocardial Blood Flow

Regional myocardial blood flow measurements were performed at rest and during maximal coronary vasodilation by use of multiple fluorescent colored microspheres (Interactive Medical Technologies, West Los Angeles, Calif.) and quantified by the reference sample technique (Heymann Mass., et al., *Prog Cardiovasc Dis* 1977;20:55-79). Fluorescent microspheres (0.8 ml, 5 \times 10⁶ microspheres/ml, 15 μ m diameter in a saline suspension with 0.01% Tween 80) were injected into the left atrium via a 6F Judkins left 3.5 diagnostic catheter. Maximal coronary vasodilation was induced by infusing adenosine at a constant rate of 140

µg/kg/min (Fujisawa USA, Deerfield, Ill.) into the left femoral vein over a period of 6 minutes. During the last 2 minutes of the infusion, microsphere injection and blood reference withdrawal were undertaken in identical fashion to the rest study.

Following completion of the perfusion assessment, animals were sacrificed with an overdose of sodium pentobarbital and KCL. Hearts were harvested, flushed with Ringer Lactate, perfusion-fixed for 10–15 minutes, and subsequently immersion-fixed with 10% buffered formaldehyde for 3 days. After fixation was completed, the hearts were cut along the short axis into 7-mm thick slices. The 2 central slices were each divided into 8 similar sized wedges, which were further cut into endocardial and epicardial sub-segments. The average of 8 lateral ischemic zone and 8 septal normal zone sub-segments measurements were used for assessment of endocardial and epicardial regional myocardial blood flow. The relative collateral flow was also computed as the ratio of the ischemic zone/non ischemic zone (IZ/NIZ) blood flow.

Histopathology

To assess whether injecting BM aspirate via the use of an injection catheter was associated with mechanical cell damage, standard BM smears were prepared before and after propelling the freshly filtered ABM aspirate through the needle using similar injecting pressure as in the in-vivo study. Morphological assessment was performed by an independent experienced technician who was blinded to the study protocol.

Histopathology assessment was performed on sampled heart tissue. In the pilot study, 7-mm thick short-axis slices were examined under UV light to identify fluorescent-tagged areas. Each identified area was cut into 3 full thickness adjacent blocks (central, right and left) that were immersion-fixed in 10% buffered formaldehyde. Subsequently, each such block was cut into 3 levels, of which 2 were stained with Hematoxylin and Eosin (H&E) and one with PAS. In addition, one fresh fluorescent-labeled tissue block was obtained from the ischemic region of each animal and was embedded in OCT compound (Sakura Finetek USA Inc., Torrance, Calif.) and frozen in liquid nitrogen. Frozen sections of these snap-frozen myocardial tissue were air dried and fixed with acetone. Immunoperoxidase stain was performed with the automated Dako immuno Stainer (Dako, Carpinteria, Calif.). The intrinsic peroxidase and non-specific uptake were blocked with 0.3% hydrogen peroxide and 10% ovo-albumin. Monoclonal mouse antibody against CD-34 (Becton Dickinson, San Jose, Calif.) was used as the primary antibody. The linking antibody was a biotinylated goat anti-mouse IgG antibody and the tertiary antibody was streptavidin conjugated with horse reddish peroxidase. Diaminobenzidine (DAB) was used as the chromogen and the sections were counterstained with 1% methylgreen. After dehydration and clearing, the slides were mounted and examined with a Nikon Labphot microscope.

In the efficacy study, full-thickness, 1.5 square centimeter sections from the ischemic and non-ischemic regions were processed for paraffin sections. Each of the samples was stained with H&E, Masson's trichrome, and factor VIII related antigen. The immunoperoxidase stained slides were studied for density of endothelial cell population and vascularization. The latter was distinguished from the former by the presence of a lumen. Vascularity was assessed using 5 photomicrographs samples of the factor VIII stained slides taken from the inner half of the ischemic and non-ischemic myocardium. Density of endothelial cells was assessed using digitized images of the same photomicrographs. The

density of the endothelial population was determined by Sigma-Scan Pro morphometry software using the intensity threshold method. The total endothelial area for each sample as well as for each specimen were obtained along with the relative percent endothelial area (endothelial area/area of the myocardium studied). The total endothelial area was also calculated as the relative percent of the non-infarcted (viable) area of the myocardium studied. The trichrom stained sections were digitized and the area occupied by the blue staining collagen as well as the total area of the section excluding the area occupied by the epicardium (which normally contained collagen) were measured using Sigma-Scan Pro. The infarcted area was then calculated as the area occupied by the blue staining.

Procedural Data

Intra-myocardial injections either with ABM or placebo were not associated with any acute change in mean blood pressure, heart rate or induction of arrhythmia. All hemodynamic parameters were comparable between the two groups. Pair-wise comparison showed similar hemodynamic parameters within each group in the index compared to the follow-up procedure except for higher initial mean arterial blood pressure at follow-up in the control group ($P=0.03$) with no subsequent differences during pacing or adenosine infusion.

Myocardial Function

Regional myocardial function assessment is shown in Table I below. Pre-intervention relative fractional wall thickening, expressed as ischemic zone to non-ischemic zone (IZ/NIZ) ratio $\times 100$, at rest and during pacing, was similar between groups ($P=0.86$ and 0.96 , respectively). At 4 weeks following the intra-myocardial injection of ABM, improved regional wall thickening occurred at rest and during pacing, which was due to an ~50% increase in wall thickening of the collateral-dependent ischemic lateral wall. No significant changes were observed in the control animals, although a trend towards improvement in wall thickening was noted in the ischemic area during pacing at follow-up.

TABLE I

Regional Contractility of the Ischemic Wall			
	Baseline	Follow-up	P
<u>Rest</u>			
ABM (%)	60 \pm 32	83 \pm 21	0.04
Control (%)	64 \pm 46	69 \pm 48	0.74
<u>Pacing</u>			
ABM (%)	36 \pm 43	91 \pm 44	0.056
Control (%)	37 \pm 56	65 \pm 56	0.23

ABM indicates autologous bone marrow.

Myocardial Perfusion Data

Regional myocardial perfusion assessment is shown in Table II below. There were no differences between the treated and control groups in the pre-intervention relative transmural myocardial perfusion, IZ/NIZ, at rest and during adenosine infusion ($P=0.42$ and 0.96 , respectively). At 4 weeks following ABM injection, relative regional transmural myocardial perfusion at rest and during pacing improved significantly. This was due to an absolute improvement in myocardial perfusion in the ischemic zone both at rest (an increase of 57%, $P=0.08$) and during adenosine infusion (37%, $P=0.09$), while no significant changes were noted in absolute flow to the non-ischemic zone either at rest (increase of 35%, $P=0.18$) or during adenosine infusion (in-

crease of 25%, $P=0.26$). The increase in regional myocardial blood flow found in the ischemic zones consisted of both endocardial (73%) and epicardial (62%) regional improvement at rest, with somewhat lesser improvement during adenosine infusion (40% in both zones). At 4 weeks, the control group showed no differences in transmural, endocardial or epicardial perfusion in the ischemic and non-ischemic zones compared to pre-intervention values.

TABLE II

Regional Myocardial Perfusion			
	Baseline	Follow-up	P
<u>Rest</u>			
ABM (%)	83 \pm 12	98 \pm 14	0.001
Control (%)	89 \pm 9	92 \pm 0.1	0.43
<u>Adenosine</u>			
ABM (%)	78 \pm 12	89 \pm 18	0.025
Control (%)	77 \pm 5	78 \pm 11	0.75

ABM indicates autologous bone marrow.

Histopathology and Vascularity Assessment

Assessment of BM smears before and after passing the filtrated aspirate through the injecting catheter revealed normal structure, absence of macro-aggregates and no evidence of cell fragments or distorted cell shapes. Histopathology at day 1 following injections revealed acute lesions characterized by fibrin and inflammatory tract with dispersed cellular infiltration. The infiltrate was characterized by mononuclear cells that morphologically could not be differentiated from a BM infiltrate. Cellularity was maximal at 3 and 7 days and declined subsequently over time. At 3 weeks, more fibrosis was seen in the 0.5 ml injection-sites compared to the 0.2 ml. CD-34 immunostaining, designed to identify BM-derived progenitor cells, was performed in sections demonstrating the maximal cellular infiltrate. Overall, it was estimated that 4–6% of the cellular infiltrate showed positive immunoreactivity to CD-34.

The ischemic territory in both groups was characterized by small areas of patchy necrosis occupying overall <10% of the examined ischemic myocardium. The non-ischemic area revealed normal myocardial structure. Changes in the histomorphometric characteristics of the two groups were compared. There were no differences in the total area occupied by any blood vessel as well as the number of blood vessels >50 μ m in diameter. However, comparison of the total areas stained positive for factor VIII (endothelial cells with and without lumen) in the ischemic versus the non-ischemic territories revealed differences between the 2 groups. In the ABM group, the total endothelial cell area in the ischemic collateral-dependent zone was 100% higher than that observed in the non-ischemic territory (11.6 \pm 5.0 vs. 5.7 \pm 2.3% area, $P=0.016$), whereas there was no significant difference in the control group (12.3 \pm 5.5 vs. 8.2 \pm 3.1% area, $P=0.11$). However, other parameters of vascularity, including % area occupied by any blood vessel and number of blood vessels >50 μ m were similar in the ischemic and non-ischemic territories in both groups.

The Effect of Autologous Bone Marrow Stimulated in vivo by Pre-Administration of GM-CSF in Animal Model of Myocardial Ischemia

Chronic myocardial ischemia was created in 16 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. At four weeks minus 3 days after ameroid implantation, 8 animals underwent subcutaneous injection of GM-CSF for 3 consecutive days (dose 10 μ g/kg per day) followed (on the fourth day and exactly 4 weeks after ameroid implantation) by transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 8 control animals without GM-CSF stimulation were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone \times 100) improved in ABM-treated pigs but not in controls (ABM: 85 \pm 11 vs 72 \pm 16 at rest, $P=0.026$; 83 \pm 18 vs 64 \pm 19 during adenosine, $P=0.06$; Controls: 93 \pm 10 vs 89 \pm 9 at rest, $P=0.31$; 73 \pm 17 vs 75 \pm 8 during adenosine, $P=0.74$). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 93 \pm 33 vs 63 \pm 27 at rest, $P=0.009$; 84 \pm 36 vs 51 \pm 20 during pacing, $P=0.014$, Controls: 72 \pm 45 vs 66 \pm 43 at rest, $P=0.65$; 70 \pm 36 vs 43 \pm 55 during pacing, $P=0.18$).

The results indicate that catheter-based transendocardial injection of ABM pre-stimulated in vivo by GM-CSF administered systemically for 3 days, can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Example 6

Treatment of a Human Patient

Bone marrow (~5 ml) will be aspirated from the iliac crest at approximately 60 minutes prior to initiation of the cardiac procedure using preservative-free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow will be immediately macro-filtered using 300 μ and 200 μ stainless steel filters, sequentially. An experienced hematologist will perform the procedure under sterile conditions. The bone marrow smear will be evaluated to confirm a normal histomorphology of the bone marrow preparation.

Any of several procedures for delivery of an agent to the myocardium can be used. These include direct transepical delivery, as could be achieved by a surgical approach (for example, but not limited to, a transthoracic incision or transthoracic insertion of a needle or other delivery device, or via thoracoscopy), or by any of several percutaneous procedures. Following is one example of percutaneous delivery. It should be emphasized that the following example is not meant to limit the options of delivery to the specific catheter-based platform system described in the example—any catheter-based platform system can be used.

Using standard procedures for percutaneous coronary angioplasty, an introducer sheath of at least 8F is inserted in the right or left femoral artery. Following insertion of the arterial sheath, heparin is administered and supplemented as

needed to maintain an ACT for 200–250 seconds throughout the LV mapping and ABM transplantation portion of the procedure. ACT will be checked during the procedure at intervals of no longer than 30 minutes, as well as at the end of the procedure to verify conformity with this requirement.

Left ventriculography is performed in standard RAO and/or LAO views to assist with guidance of NOGA-STAR™ and injection catheters, and an LV electromechanical map is obtained using the NOGA-STAR™ catheter. The 8F INJECTION-STAR catheter is placed in a retrograde fashion via the femoral sheath to the aortic valve. After full tip deflection, the rounded distal tip is gently prolapsed across the aortic valve and straightened appropriately once within the LV cavity.

The catheter (incorporating an electromagnetic tip sensor) is oriented to one of the treatment zones (e.g. anterior, lateral, inferior-posterior or other). Utilizing the safety features of the NOGA™ system, needle insertion and injection is allowed only when stability signals will demonstrate an LS value of <3. A single injection of 0.2 cc of freshly aspirated ABM will be delivered via trans-endocardial approach to the confines of up to two treatment zones with no closer than 5 mm between each injection site. The density of injection sites will depend upon the individual subject's LV endomyocardial anatomy and the ability to achieve a stable position on the endocardial surface without catheter displacement or premature ventricular contractions (PVCs).

That freshly aspirated autologous bone marrow transplanted into ischemic myocardium is associated with improved collateral flow without adverse effects may be of clinical importance for several reasons. The methodology reflected above took advantage of the natural capability of the bone marrow to induce a localized angiogenic response in an effective and apparently safe manner. Such an angiogenic strategy would probably be less costly than many others currently being tested. It would also avoid potential toxicity-related issues that are remote but definite possibilities with various gene-based approaches using viral vectors.

The invention is based on the concept that autologous bone marrow may be an optimal source for cellular (an example would be endothelial progenitor cells, but the invention is not limited to such cells as many other cells in the bone marrow may contribute importantly to the angiogenic effect) and secreted, e.g., angiogenic growth factors, elements necessary to promote new blood vessel growth and restore function when transferred to another tissue, such as ischemic heart or peripheral limbs. A patient's own bone marrow can be used as the key therapeutic source to induce therapeutic angiogenesis and/or myogenesis in ischemic tissues, e.g., heart muscle and/or ischemic limb, with compromised blood perfusion due to arterial obstructions. The patient's own bone marrow is aspirated, i.e., autologous bone marrow donation, processed, and injected directly into ischemia and/or adjacent non-ischemic tissue, e.g., heart muscle and/or ischemic limb, to promote blood vessel growth.

The autologous bone marrow and/or bone marrow products are injected into the heart muscle, e.g., the myocardium, by use of either a catheter-based trans-endocardial injection approach or a surgical (open chest or via thoracoscopy) trans-epicardial thoracotomy approach. Those two delivery strategies can be used to achieve the same therapeutic goal by promoting the incorporation and integration of angiogenic bone marrow elements in the target organ tissue, e.g., heart muscle and/or ischemic limb.

According to the invention, effective amounts of autologous bone marrow are administered for treatment. As would

be appreciated by experienced practitioners, the amount administered will depend upon many factors, including, but not limited to, the intended treatment, the severity of a condition being treated, the size and extent of an area to be treated, etc. With regard to treatment according to the invention, a representative protocol would be to administer quantities of from about 0.2 to about 0.5 ml of autologous bone marrow in each of from about 12 to about 25 injections, for a total of from about 2.4 to about 6 ml of autologous bone marrow being administered. Each dose administered could preferably comprise from about 1 to about 2 percent by volume of heparin or another blood anticoagulant, such as coumadin. When the autologous bone marrow has been cultured or stimulated and/or is being administered in combination with other pharmaceuticals or the like, the quantity of autologous bone marrow present should be approximately the same in each dose and/or the total of the autologous bone marrow administered should be about the same as described above. It is believed that the total number of cells of autologous bone marrow administered in each treatment should be on the order of from about 10^7 to 5×10^8 .

Optimization of angiogenic gene expression requires the co-administration of various angiogenic stimulants with the autologous bone marrow. Thus, according to the invention autologous bone marrow transplantation is injected either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Examples of these "combined" agents (although not limited to these agents) are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP 1), EPAS1, or Hypoxia Inducible Factor-1 (HIF-1). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. An example of an intervention that may enhance bone production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. This intervention can be used alone with bone marrow, or in combination with any of the factors outlined above. These optimization strategies are designed to increase the production of vascular endothelial growth factor (VEGF) expression and/or other cytokines with angiogenic activity prior to the direct injection of the bone marrow into the heart or any peripheral ischemic tissue. In a broad sense, the invention comprises intramyocardial injection of autologous bone marrow with any agent that would become available to cause stimulation of bone marrow and/or ex-vivo or in vivo stimulation of any angiogenic growth factor production by the bone marrow or its stromal microenvironment.

Delivery to patients will vary, dependent upon the clinical situation. For example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its border zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesis and/or myogenesis. For

example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its borderline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesis and/or myogenesis. This procedure will involve the use of a bone marrow aspiration procedure, bone marrow harvesting and processing, followed by the use of the autologous bone marrow or its elements (growth factors and/or cellular elements being isolated from the patient's own bone marrow), with or without any ex-vivo stimulation of its delivery forms, to be injected into the ischemic or non ischemic myocardium and/or peripheral ischemic tissue (such as limb ischemia). The bone marrow will be kept in standard anti-coagulation/anti-aggregation solution (containing sodium citrate and EDTA) and kept in 4° C. in sterile medium until the time of its use.

Upon its use, the bone marrow will be filtered to avoid injecting remaining blood clots or macroaggregates into the target tissue.

The bone marrow, with or without a stimulatory agent in any of its delivery forms, or with or without having been transfected with a vector carrying a transgene that is designed to enhance the angiogenesis effect of the bone marrow, will be injected into the heart muscle, i.e., in therapeutic myocardial angiogenesis or therapeutic myogenesis, using either any catheter-based trans-endocardial injection device or via a surgical (open chest) trans-epicardial thoracotomy approach, or any other approach that allows for transepical delivery. In the case of treatment of limb ischemia the bone marrow will be transferred by a direct injection of the bone marrow or its elements, with or without ex-vivo or in vivo stimulation in any of its delivery forms, into the muscles of the leg.

The volume of injection per treatment site will probably range between 0.1–5.0 cc per injection site, dependent upon the specific bone marrow product and severity of the ischemic condition and the site of injection. The total number of injections will probably range between 1–50 injection sites per treatment session.

The preceding specific embodiments are illustrative of the practice of the invention. It is to be understood, however, that other expedients known to those skilled in the art or disclosed herein, may be employed without departing from the spirit of the invention or the scope of the appended claims.

We claim:

1. A method of enhancing collateral blood vessel formation in a subject comprising directly administering to sites in heart or limb tissue an effective amount of autologous bone marrow aspirate to induce collateral blood vessel formation in the tissue.

2. The method of claim 1, wherein the autologous bone marrow aspirate is injected.

3. The method of claim 1, wherein the autologous bone marrow aspirate is injected intramyocardially.

4. The method of claim 2 wherein the wherein the autologous bone marrow aspirate is injected trans-epicardially or trans-endocardially.

5. The method of claim 4, wherein the trans-endocardial approach is via a catheter.

6. The method of claim 1, wherein the autologous bone marrow aspirate has been stimulated while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

7. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated by contact with one or more angiogenesis stimulating cytokines secreted therefrom while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

8. The method of claim 1, wherein the autologous bone marrow aspirate further comprises Monocyte Chemoattractant Protein 1 (MCP-1) or Vascular Endothelial Growth Factor (VEGF).

9. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated ex vivo in culture by transient exposure to hypoxia.

10. The method of claim 1, wherein the autologous bone marrow aspirate is administered in combination with one or more agent selected from a pharmacological drug or protein that enhances bone marrow production of angiogenic growth factors selected to promote endothelial cell proliferation, migration, or blood vessel formation.

11. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are administered together.

12. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are combined ex vivo prior to administration.

13. The method of claim 12, wherein the autologous bone marrow aspirate has been stimulated ex vivo in conditioned medium, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

14. The method of claim 1, wherein the autologous bone marrow aspirate is administered to ischemic tissue.

15. The method of claim 12, further comprising culturing the autologous bone marrow aspirate to form conditioned medium containing bone marrow cells and endogenously secreted angiogenic cytokines and injecting the composition into ischemic heart tissue.

* * * * *

EVIDENCE APPENDIX

ITEM NO. 18

**American Heart Association 2004 article titled,
“Endothelial Progenitor Cells: More than an Inflammatory Response?”
cited by Appellant as Exhibit C
in the Response filed November 28, 2007**

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Ton J. Rabelink, Hetty C. de Boer, Eelco J.P. de Koning and Anton-Jan van
Zonneveld

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Endothelial Progenitor Cells: More Than an Inflammatory Response?

Ton J. Rabelink, Hetty C. de Boer, Eelco J.P. de Koning, Anton-Jan van Zonneveld

Abstract—The formation of new capillaries (angiogenesis) may be of clinical importance in facilitating reperfusion and regeneration of hibernating cardiac tissue after myocardial infarction and in microvascular ischemia. Evidence is accumulating that as part of the response to hypoxia, bone marrow-derived circulating endothelial progenitor cells (CEPs) are mobilized and subsequently differentiate into proper endothelial cells. There are also indications that such CEPs can facilitate endothelial repair and angiogenesis in vivo. It is not clear yet, however, whether these CEPs are essential for these adaptive processes or what the relative contribution of CEP is compared with that of other mononuclear inflammatory cells that are mobilized to areas of ischemia. Moreover, there are still many uncertainties about how cardiovascular risk factors alter CEP function. Particularly when therapeutically mobilizing CEPs, a further understanding of this issue is essential to assess the risk of potentially harmful side effects of altered CEP function. (*Arterioscler Thromb Vasc Biol.* 2004;24:834–838.)

Key Words: angiogenesis ■ atherosclerosis ■ endothelial progenitor

Over the past 7 years, the discovery of the phenomenon that mononuclear cells in peripheral blood have the potential to differentiate into endothelial cells *ex vivo* as well as *in vivo* has opened up a new field of cardiovascular research.^{1,2} It thus appears that such endothelial progenitor cells (EPCs) can be used therapeutically to restore damaged endothelium.^{3,4} They can also incorporate into the endothelial monolayer and stimulate proliferation of neighboring endothelial cells, thus inducing the formation of new blood vessels.^{5,6} Although the biology is not really understood, several pilot studies have suggested beneficial effects of infusion of mononuclear cells after myocardial infarction in animal models^{7–10} and in humans.¹¹ The aim of the current review is to discuss some of the potential regulatory mechanisms involved in this phenomenon. In particular, we address the question of whether these progenitor cells are a specific subpopulation with stem cell properties or whether these cells merely reflect plasticity of the normal inflammatory response that occurs in occlusive vascular disease. Ultimately, understanding this biology will be a critical success factor for bringing progenitor cell therapy into the clinical arena.

Vascular Occlusion Leads to Inflammation

In the adult, vessels grow either via capillary sprouting (angiogenesis) or via remodeling of pre-existing arteriolar connections into collateral vessels (arteriogenesis).^{12,13} Both processes occur on occlusion of a vessel, thus improving blood delivery and local perfusion of ischemic tissue. The regulation of the cellular processes involved in arteriogenesis

has recently been reviewed in this journal.¹³ In this review, we focus on the contribution of bone marrow-derived progenitor cells in the (microcirculatory) angiogenic response to ischemia, a process referred to as (postnatal) vasculogenesis. The endothelial cell is key in initiating vasculogenesis. First, on vascular occlusion, endothelial cells will sense altered shear stress. Recent studies have shown that at low shear stress or oscillatory shear stress, endothelial cells typically will increase the expression of pro-oxidant enzymes, such as NADH oxidase,¹⁴ and reduce the expression of anti-oxidant enzymes such as manganese superoxide dismutase,¹⁵ thioredoxin reductase, and glutathione reductase.¹⁶ As a result, altered shear stress during vascular occlusion will result locally in increased redox signaling.¹⁷ One of the activated transcription factors is NF- κ B, which plays a central role in the inflammatory response.¹⁸ NF- κ B activation in the endothelium results in the expression of adhesion molecules and the release of chemotactic factors for inflammatory cells.¹⁹ In agreement, *in situ* nuclear translocation of NF- κ B has been found in the vessel wall near regions of disturbed blood flow, like bifurcations, curvatures, and branching points.²⁰ The second parameter that endothelial cells sense during vascular occlusion is hypoxia. Basically, the endothelial cell is equipped with 2 systems to sense such hypoxia. One is the transcription factor HIF-1 α .²¹ Under normoxic conditions, this transcription factor will be hydroxylated on a conserved prolyl residue in a reaction with molecular oxygen. The hydroxylated prolyl group allows the Von Hippel-Lindau protein to bind and polyubiquitinate the molecule, thereby

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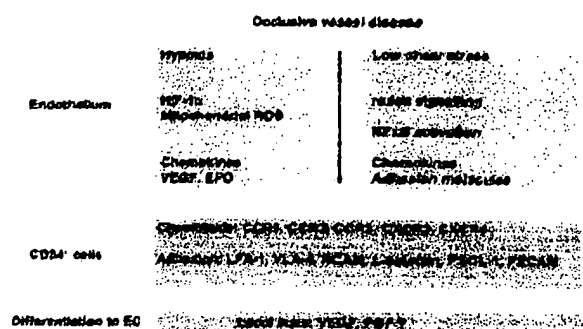


Figure 1. In atherosclerotic vascular disease, the endothelial cells will be exposed to hypoxia and altered shear stress. Both mechanisms induce redox signaling in the endothelial cell, with subsequent release of chemokines and surface expression of leukocyte adhesion molecules. The CD34⁺ hematopoietic stem cell is equipped with several receptors, which allow for chemotaxis, and with ligands for adhesion. Local cues such as the concentration of VEGF and basic FGF probably determine differentiation into endothelial-like cells.

marking HIF-1 α for destruction by the proteasome. However, in hypoxia, this hydroxyproline is reduced, allowing HIF-1 α to accumulate and translocate to the nucleus to activate transcription of genes such as VEGF, EPO, and inducible NOS. The other oxygen-sensing mechanism in the endothelial cell is located in the mitochondria where, under normoxic conditions, oxidative phosphorylation occurs.²² This reaction is constitutively inhibited by nitric oxide that is also produced in the mitochondria. When hypoxia occurs, nitric oxide starts to dominate and reduces electron transport toward its final acceptor, complex IV.²³ As a result, intermediate radical products such as hydrogen peroxide and reactive nitrogen species are produced by the mitochondria, again leading to redox signaling and subsequent activation of the endothelial cell. Taken together, altered shear stress and hypoxia will result in redox-mediated endothelial cell activation and release of chemokines, cytokines, and growth factors. Thus, occlusive vascular disease inevitably results in a local inflammatory response of the endothelium (Figure 1).

Endothelial Progenitor Cells: Part of the Inflammatory Response?

For some time, it has been suggested that as a consequence of such an inflammatory response, the ensuing recruitment of monocytes are instrumental not only in inducing collateral formation but also in promoting angiogenesis.^{13,24} It is believed that paracrine release of cytokines and growth factors with known angiogenic properties, such as bFGF and TNF α , mediate these effects of monocytes on capillary sprouting.²⁵

More recently, attention has also been drawn to other vasculogenic cell populations present in the mononuclear fraction of peripheral blood that may also be recruited to the activated endothelium in response to an ischemic insult. These are referred to as pluripotent stem cell or progenitor cell populations and include the CD34⁺ hematopoietic stem cells and subpopulations of CD34⁺ mononuclear cells, and even subpopulations of the peripheral blood monocytes. Isolation of each of these subpopulations and subsequent

culture *in vitro* could give rise not only to classical circulating blood cells such as monocytes/macrophages but also to unexpected phenotypes such as endothelial cells and myocytes.^{1,26–28} Hence, the concept has evolved that vascular progenitors are recruited from the bone marrow to sites of tissue revascularization, where they participate in a paracrine way and also directly by differentiating into mature endothelial cells. In particular, the CD34⁺ hematopoietic stem cell has raised a lot of attention in this respect because of the similarities with the embryonic hemangioblast, which gives rise not only to circulating blood cell lineages but also to vascular cells.²⁹ Hematopoietic stem cells appear to be essential for angiogenesis in the mouse embryo³⁰ and, when durably engrafted in adult mice, were shown to have functional hemangioblast activity and develop into endothelial cells that participate in the neovasculature that evolved after retinal ischemia.³¹

Although bone marrow transplantation experiments have shown unequivocally that bone marrow-derived cells can also differentiate into vascular cells *in situ*, the frequency of this phenomenon and the identification of the cell type involved are still matter of debate.³² Only recently have some specific surface markers for “true” EPCs emerged from detailed studies characterizing mammalian embryogenesis and angiogenesis. Flk-1/KDR is a receptor for vascular endothelial cell growth factor (VEGFR-2), which appears to be critical for embryonic endothelial cell differentiation and vasculogenesis.³³ Also, it was reported that Flk-1-positive cells, derived from differentiated embryonic stem cells, can give rise to endothelial cells and vascular smooth muscle cells *in vitro* and *in vivo*.³⁴

Together with the essential role of Flk-1 in hematopoiesis,³⁵ these observations are consistent with the existence of a Flk-1-positive hemangioblast that serves as a common origin of endothelial cells and blood cells.³⁶ AC133 is a second early hematopoietic stem cell marker that is downregulated on differentiation and is therefore a marker for early EPCs. Indeed, AC133-positive cells from human peripheral blood were shown to differentiate into endothelial cells *in vitro*.³⁷ Using these stem cell markers, it has become clear that only a very small subset of circulating mononuclear cells in peripheral human blood stains (0.002%) positively for CD34, AC133, and Flk-1 simultaneously.³⁸ The most detailed phenotypic description of the circulating EPC (CEP) proposes the co-expression of several common endothelial and hematopoietic antigens: CD34⁺, FGFR⁺, CD38⁺, VE-cadherin⁺, c-kit⁺, CD31⁺, Flt-1, AC133⁺; in addition, it represents even a subfraction of these.³⁹

Are these CEP part of the inflammatory response on vascular occlusion and, if so, does contribution of these cells matter in view of the reported effects of the abundantly present monocytes on these processes? To play such a role, CEP should have the capacity to home exclusively on sites of angiogenesis. They should be able to attach to activated endothelium or extracellular matrix, to (trans)differentiate into an endothelial phenotype, and be able to proliferate. We recently demonstrated that CD34⁺ hematopoietic stem cells specifically home and migrate to angiogenic endothelium (unpublished observation). Although CD34⁺ cells probably

do not adhere to normal endothelium, they can attach to activated endothelium. Platelets may play an important modulating role in this attachment. Platelets can adhere to inflamed endothelial cells or to exposed extracellular matrix, where they express P-selectin and thus can provide an adhesive surface for CEP;⁴⁰ however, CD34⁺ cells express the binding determinant for P-selectin (PSGL-1).⁴¹ In particular, the issue whether endothelial progenitor cells can proliferate after homing and (trans)differentiation may be important in appreciating the *in vivo* relevance of these CEP as sources of paracrine factors and as sources for endothelial cells versus the effects that other inflammatory cells have on resident endothelium. Bone marrow transplantation experiments show low to very low percentages of *in situ* differentiation of bone marrow-derived cells into endothelial cells, making the role for CEPs as a major source of endothelial cells in the short-term perspective of these experiments less likely. However, in acute ischemic events such as myocardial infarction, the number of circulating CD34⁺ cells was increased and a direct correlation with plasma levels of VEGF was shown.⁴² Furthermore, in patients experiencing an acute vascular insult secondary to burns or coronary bypass grafting, a rapid increase (50×) in the number of CEP was noted within 6 to 12 hours after injury, which coincided with an elevation in VEGF level.⁴³ Clearly, increased numbers of circulating CD34⁺ cells, in combination with efficient homing and ultimately (trans)differentiation and proliferation at the site of vascular injury, may increase the contribution of these progenitor cells relative to other inflammatory cells. This phenomenon can be used therapeutically by artificially increasing the number of circulating progenitor cells in conditions such as ischemia. VEGF, basic fibroblast growth factor, angiopoietin-1, placental growth factor, and stromal cell-derived growth factor-1 have all been shown to induce EPC mobilization and recruitment.^{2,44} What most of these factors have in common is that they stimulate the Akt/PKB pathway; evidence is accumulating that the Akt/PKB pathway plays a central role in stem cell recruitment and survival.⁴⁵ Activation of this pathway may also explain some of the effect of statins on *in vivo* (re)endothelialization, an effect that appears, at least partly, dependent on incorporation of bone marrow-derived cells into the endothelial cell monolayer.^{4,46} Because EPCs are thought to be derived from the CD34⁺ hematopoietic stem cell, an additional method to increase circulating EPCs is to use stem cell mobilizing factors, such as stem cell factor and granulocyte-macrophage colony-stimulating factor. In experimental myocardial infarction^{47,48} and the ischemic hindlimb,⁴⁹ it was found that during therapy with such cytokines, circulating EPCs were mobilized into the ischemic regions and augmented neovascularization of ischemic tissue. Recently, a stimulatory effect of erythropoietin (Epo) has also been described on EPC recruitment and angiogenesis in the mouse model of inflammation and ischemia-induced neovascularization.⁵⁰ Also, in renal anemia patients, recombinant Epo markedly increased the number of CD34⁺ cells in the circulation.⁵¹ Although these observations are exciting, it should be noticed that the beneficial effects of mobilizing CEPs on ischemia were observed in otherwise healthy animals. In disease states or in

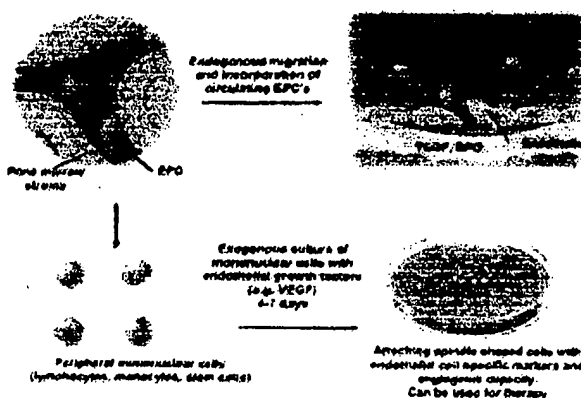


Figure 2. Endothelial progenitor cells are referred to as the endogenous CD34⁺-derived cells that can incorporate in damaged endothelium or hypoxic tissue (upper part of the figure) as well as the *ex vivo* expanded and differentiated mononuclear cells, which have been shown to display endothelial-specific cell markers and angiogenic capacity (lower part of the figure).

the presence of cardiovascular risk factors, mobilization of such cells may also promote the formation of potentially harmful CD34⁺-derived phenotypes such as macrophages or fibrocytes.⁵² It thus appears that cardiovascular risk factors may shift the balance between cells that can induce repair and neovascularization toward cells that contribute to a harmful inflammatory reaction. In addition, nitric oxide availability appears to be essential for mobilization of circulating EPCs from the bone marrow stroma.⁵³ Strategies that allow the beneficial side of inflammation such as the endogenous capacity to form endothelial-like cells while at the same time reducing differentiation of harmful cellular phenotypes by drugs that enhance nitric oxide bioavailability or activate the Akt/PKB signaling pathway (eg, statin therapy^{54,55}) may therefore prove to be even more useful than mobilizing or infusing progenitor cells.

Attaching Cells: The Other EPC

The low numbers of CD34⁺ CEP (100 to 500 per mL blood) are in sharp contrast with the relatively large numbers of attached cells that are obtained (~100 000 from 1 mL blood) after culturing the blood mononuclear cell fraction on fibronectin or gelatin for 4 days in the presence of endothelial growth factors and that, unfortunately, often also are referred to as "EPC" (Figure 2). These spindle-shaped attaching cells (hereafter referred to as AT cells) exhibit endothelial characteristics such as the potential to take-up acetylated LDL and expression of endothelial markers such as ULEX and von Willebrand factor.^{1,42} This remarkable plasticity of cells present in the AT cell cultures cannot be explained by the presence of a few co-isolated CEPs and more likely originate from a more abundant circulating mononuclear cell type, such as monocytes.^{24,56–59} The concept that has developed over the years is that the number of these AT cells quantitatively reflect subpopulations within the blood mononuclear cells that have the potential to differentiate into an endothelial phenotype *in vivo*. Interestingly, the number of AT cells is reduced in patients with cardiovascular risk factors.⁶⁰ Recently, this reduction has been related to intermedi-

ate endpoints of cardiovascular disease, such as impaired flow-mediated dilation.⁶¹ However, one has to realize that the AT cell cultures are an in vitro phenomenon and thus subject to methodological influences. For example, increased expression of matrix adhesion molecules such as the vitronectin receptor $\alpha_3\beta_3$ in mononuclear cells (eg, by statin therapy) may yield higher numbers of AT cells in culture conditions in which vitronectin is used as an adhesive surface.^{4,62} Thus, when certain adhesion receptors are altered in mononuclear cells, either disease- or therapy-related, then this will be noticed only when the mononuclear cells are cultured on the relevant adhesive protein(s). To date, ~6 different adhesive surfaces have been used in the EPC culture assay: FN, FN plus gelatin, gelatin, VN plus gelatin, FN plus collagen, and collagen type I. To what extent these different surfaces have led to conflicting interpretations is not clear. However, we feel that the concept that the number of AT cells quantitatively reflects the number of circulating EPCs has to be carefully interpreted. Nevertheless, these AT cells appear to offer spectacular therapeutic opportunities. Intravenous infusion of these AT cells in animal models of ischemia results in homing of these cells to the ischemic tissue and augmentation of neovascularization.^{4,9,63} These effects are specific, because infusion with mature endothelial cells had no such an effect.⁵ Based on these observations, clinical studies have been initiated such as the TOP-CARE study, to investigate whether infusion of autologous expanded AT cells in patients with myocardial infarction reduces ischemic injury.¹¹

In conclusion, evidence is accumulating that, as part of the response to hypoxia, circulating endothelial progenitor cells are mobilized from the bone marrow and subsequently differentiate into proper endothelial cells. There are also indications that such CEPs can facilitate endothelial repair and angiogenesis in vivo. It is not clear yet, however, whether CEPs are essential for these adaptive processes or what the relative contribution of CEP is compared with that of other mononuclear inflammatory cells. Moreover, there are still many uncertainties about how cardiovascular risk factors modulate CEP function. Particularly when therapeutically mobilizing CEPs, a further understanding of this issue is essential to assess the risk of transdifferentiation of CEPs to potentially pro-atherogenic inflammatory cells.^{64,65}

Acknowledgments

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References

- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;273:964-967.
- Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med*. 2003;9:702-712.
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Wagner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res*. 1999;85:221-228.
- Walter DH, Rittig K, Bahlmann FH, Kirchmaier R, Silver M, Murrayama T, Mubumura H, Losordo DW, Asahara T, Isner JM. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation*. 2002;105:3017-3024.
- Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A*. 2000;97:3422-3427.
- Schatteman GC, Hantson HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest*. 2000;106:571-578.
- Kamihata H, Matsubara H, Nishio T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imazumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046-1052.
- Kamihata H, Matsubara H, Nishio T, Fujiyama S, Amano K, Iba O, Imada T, Iwasaka T. Improvement of collateral perfusion and regional function by implantation of peripheral blood mononuclear cells into ischemic hibernating myocardium. *Arterioscler Thromb Vasc Biol*. 2002;22:1804-1810.
- Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JL, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634-637.
- Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Iftescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430-436.
- Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dohert N, Guruswamy S, Aicher A, Urbich C, Martin H, Hoecker D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009-3017.
- Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653-660.
- Schaper W, Scholz D. Factors regulating arteriogenesis. *Arterioscler Thromb Vasc Biol*. 2003;23:1143-1151.
- De Keulener GW, Chappell DC, Ishizaka N, Norum RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res*. 1998;82:1094-1101.
- Topper JN, Cai J, Falb D, Gimbrone MA Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A*. 1996;93:10417-10422.
- Hojo Y, Saito Y, Tanimoto T, Hoefen RJ, Baines CP, Yamamoto K, Haendeler J, Asmis R, Berk BC. Fluid shear stress attenuates hydrogen peroxide-induced c-Jun NH2-terminal kinase activation via a glutathione reductase-mediated mechanism. *Circ Res*. 2002;91:712-718.
- Thamkral VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol*. 2000;279:L1005-L1028.
- Bacuerle PA. IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell*. 1998;95:729-731.
- Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 1998;16:225-260.
- Hajra L, Evans AI, Chen M, Myduk SJ, Collins T, Cybulsky MJ. The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation. *Proc Natl Acad Sci U S A*. 2000;97:9052-9057.
- Safran M, Kaelin WG Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. *J Clin Invest*. 2003;111:779-783.
- Chandel NS, Schumacker PT. Cellular oxygen sensing by mitochondria: old questions, new insights. *J Appl Physiol*. 2000;88:1880-1889.
- Mosca S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat Rev Mol Cell Biol*. 2002;3:214-220.
- Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol*. 1994;55:410-422.
- Azra M, Ito WD, Sobola D, Winkler B, Schaper J, Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest*. 1998;101:40-50.
- Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsas HP, Muller R, Havemann K. Endothelial-like cells derived from human CD14 positive monocytes. *Differentiation*. 2000;65:287-300.

27. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Ertan ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107:1395-1402.
28. Zhao Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2003;100:2426-2431.
29. Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Douarin NM. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci U S A*. 1997;94:5141-5146.
30. Takakura N, Watanabe T, Suecoba S, Yamada Y, Noda T, Ito Y, Satake M, Suda T. A role for hematopoietic stem cells in promoting angiogenesis. *Cell*. 2000;102:199-209.
31. Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE, Peck AB, Scott EW. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med*. 2002;8:607-612.
32. Vorwinkler R, Ziegheoffer T, Heil M, Kottin S, Bräuer G, Mehling T, Haberberger R, Clauss M, Gammann A, Schaper W, Seeger W. Circulating vascular progenitor cells do not contribute to compensatory lung growth. *Circ Res*. 2003;93:372-379.
33. Shalaby F, Ho J, Stanford WL, Fischer KD, Schuh AC, Schwartz L, Bernstein A, Roessant J. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell*. 1997;89:981-990.
34. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature*. 2000;408:92-96.
35. Ziegler BL, Vallieri M, Porada GA, De Maria R, Muller R, Masella B, Gabbiani M, Caselli J, Pelosi E, Böck T, Zanetti ED, Peschle C. KDR receptor: a key marker defining hematopoietic stem cells. *Science*. 1999;285:1553-1558.
36. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development*. 1998;125:725-732.
37. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otto M, Schuch G, Schaffhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hoesfeld DK, Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood*. 2000;95:3106-3112.
38. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood*. 2000;95:952-958.
39. Burger PE, Coetzee S, McKeehan WL, Kan M, Cook P, Fan Y, Suda T, Heibel RP, Novitsky N, Müller WA, Wilson EL. Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. *Blood*. 2002;100:3527-3535.
40. Janowska-Wieczorek A, Majka M, Kijowski J, Baj-Krzyworozek M, Reza R, Turner AB, Ratajczak J, Emerson SG, Kowalska MA, Ratajczak MZ. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood*. 2001;98:3143-3149.
41. Hidalgo A, Weiss LA, Frenette PS. Functional selectin ligands mediating human CD34(+) cell interactions with bone marrow endothelium are enhanced postnatally. *J Clin Invest*. 2002;110:559-569.
42. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation*. 2001;103:2776-2779.
43. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res*. 2001;88:167-174.
44. Szmitko PE, Fedak PW, Weiss RD, Stewart DJ, Kutryk MJ, Verma S. Endothelial progenitor cells: new hope for a broken heart. *Circulation*. 2003;107:3093-3100.
45. Maingi AA, Noisoux N, Koo D, He H, Ryzanski M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med*. 2003;9:1195-1201.
46. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tlemann M, Rutten U, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest*. 2001;108:391-397.
47. Norol F, Merlet P, Isnard R, Sobillon P, Boenke N, Cailliot C, Carlier C, Ribeiro M, Charlotte F, Pradeau P, Mayol JF, Peinaquin A, Drouot M, Safsafi K, Vernant JP, Herodot P. Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. *Blood*. 2003;102:4361-4368.
48. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A*. 2001;98:10344-10349.
49. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med*. 1999;5:434-438.
50. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiological stimulus for endothelial progenitor cell mobilization. *Blood*. 2003;102:1340-1346.
51. Bahlmann FH, DeGroot K, Duckert T, Niemczyk B, Bahlmann E, Boehm SM, Haller H, Fliser D. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. *Rapid Communication. Kidney Int*. 2003;64:1648-1652.
52. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol*. 2001;166:7556-7562.
53. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med*. 2003;9:1370-1376.
54. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, Dimmeler S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation*. 2001;103:2885-2890.
55. Llevadot J, Murasawa S, Kurishiki Y, Uchida S, Maruda H, Kawamoto A, Walsh K, Isner JM, Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J Clin Invest*. 2001;108:399-405.
56. Harnes M, Jiao C, Hanlon HD, Hartley RS, Schattman GC. CD34(+) blood-derived human endothelial cell progenitors. *Stem Cells*. 2001;19:304-312.
57. Schmeisser A, Garlicks CD, Zhang H, Eskafi S, Gruffy C, Ludwig J, Strasser RH, Daniel WG. Monocytes coexpress endothelial and macrophage lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovasc Res*. 2001;49:671-680.
58. Rehman J, Li J, Orbell CM, March KL. Peripheral blood 'endothelial progenitor cells' are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107:1164-1169.
59. Roelmaaker MB, Vergeer M, van Zonneveld AJ, Rabelink TJ, Verhaar MC. Endothelial progenitor cells: mainly derived from the monocyte/macrophage containing CD34- but also partly derived from the hematopoietic stem cell containing CD34+ mononuclear population. *Circulation*. 2003;108:1506.
60. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001;89:E1-E7.
61. Hill JM, Zalos G, Halcox JP, Schenke WH, Wacziarg MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003;348:593-600.
62. Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravenraux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res*. 2000;86:1198-1202.
63. Aicher A, Brenner W, Zahayra M, Badorff C, Massoudi S, Aemmer B, Eckey T, Henze E, Zeiher AM, Dimmeler S. Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling. *Circulation*. 2003;107:2134-2139.
64. Cyphe NM, Bunch TJ, Stalborger PG, Wang S, Simper D, Miller DV, Russell SJ, Litrow MR, Edwards WD. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. *Proc Natl Acad Sci U S A*. 2003;100:4754-4759.
65. Sata M, Saito A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makinouchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med*. 2002;8:403-409.

EVIDENCE APPENDIX

ITEM NO. 19

**Declaration of Dr. G. Robert Meger
cited by Appellant as an Exhibit
in the Amendment filed February 15, 2001**



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)
SERIAL NO.: 09/064,000) EXAMINER: Nicholas D. Lucchesi
FILED: April 21, 1998)
FOR: METHOD AND APPARATUS) GROUP ART UNIT: 3732
FOR INSTALLATION OF)
DENTAL IMPLANT)

DECLARATION OF G. ROBERT MEGER, M.D.

I G. Robert Meger declare as follows:

1. I have offices at 3333 East Camelback Road, Phoenix, Arizona 85018.
2. My Curriculum Vitae is attached hereto as Exhibit A.
3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter "235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit B. I understand that the same disclosures are contained in above patent Application Serial No. 09/064,000.
4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and resulting soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection,

through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. The materials included in Exhibit C of this Declaration illustrate that the techniques set forth in above Paragraph 4 were well known to those skilled in the medical arts prior to July 2, 1993. It is my opinion that one skilled in the medical arts armed with such knowledge would have been able to practice the invention(s) described at column 14, lines 4-61 and column 21, lines 1-26 of the '235 patent without need for resorting to undue experimentation.
6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:

2/13/01

G. Robert Meyer

G. Robert Meyer

EXHIBIT A

CURRICULUM VITAE

Exhibit A

Revised 10/2000

CURRICULUM VITAE

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Good Samaritan Regional Medical Center
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Maricopa Medical Center
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Doctor of Medicine
Creighton University
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Bachelor of Science in Chemistry
University of Notre Dame
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EXHIBIT B

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud 122 (FIG. 3) is produced. The bud 122 is placed in an opening 123 in the alveolar bone and packing material is placed around or on top of the bud 122. The size of opening 123 can vary as desired. The packing around bud 122 can comprise HAC 124, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud 122 grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud 122 to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud 122 forms and grows into a full sized tooth. Instead of forming an opening 123, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

**TECHNIQUES OF INTRODUCING
AND ACTIVATION OF GROWTH FACTORS**

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-1	<u>J Periodontol</u> , November 1991, "Effects of platelet-derived growth factor/insulin-like growth factor-1 combination on bone regeneration around titanium dental implants". Lynch S.E., et. al.	Gel carrier
C-2	<u>Nature</u> , November 28, 1991, "Electrically erodible polymer gel for controlled release of drugs". Kwon, I.C., et. al.	Possibility of multiple chemical release stimuli of gel for controlled release
C-3	<u>Acta Orthop Scand</u> , October 1991, "Dose-dependent stimulation of bone induction by basic fibroblast growth in rats". Aspenberg P., et. al.	Gel carrier
C-4	<u>Natl. Acad. Sci.</u> , November 1992, "Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells". Jackson A., et. al.	Heat activation of growth factor
C-5	<u>Transplant</u> , 1992, "Cell transplantation for myocardial repair: an experimental approach". Marelli D., et. al.	Heart injection
C-6	<u>Lasers Sur. Med.</u> , 1989, "Macrophage responsiveness to light therapy". Young, S.	Light activation
C-7	<u>J Surg. Res.</u> , May 1989, "Attachment of peptide growth factors to implantable collagen". Stompro B.E., et. al.	Absorbable carrier
C-8	<u>Clin. Orthop.</u> , February 1991, "Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite". Sato T., et. al.	Non-absorbable carrier
C-9	<u>Arch Surg.</u> , June 1989, "Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits". Hockel M., Burke J.F.	Injection

EXH. NO.	MATERIAL AND DATE	TECHNIQUE
C-10	<u>JAMA</u> , October, 1991, "Tissue transformation into bone in vivo. A potential practical application". Khouri R.K., et. al.	Injection
C-11	<u>Radiology</u> , December 1986, "An experimental evaluation of microcapsules for arterial chemoembolization". Bechtel W., et. al.	Intra Arterial capsule delivery
C-12	<u>Atherosclerosis</u> , February 1989, "Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries". Sprecher D.L., et. al.	Coronary heart catheter
C-13	<u>Int. J Cancer</u> , May 1989, "Acidic Cellular Environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF". Dullien P., et. al.	pH activation
C-14	<u>Atherosclerosis</u> , April 1990, "Endothelial cell stimulation of smooth muscle glycosamino-glycan sythesis can be accounted for by transforming growth factor beta activity". Merrilees M.J., Scott L.	Heat activation
C-15	<u>Ultrasound Med Biol</u> , 1990, "Macrophage responsiveness to therapeutic ultrasound". Young S.R., Dyson M.	Ultrasound activation
C-16	<u>Am J Physiol</u> , September 1989, "Mitogenic signals for thrombin in mesangial cells: regulation of phossholipase C and PDGF genes". Schultz P.J., et. al.	Enzyme activation
C-17	<u>J Burn Cure Rehabil</u> , July-August, 1991, "Weak direct current accelerates split-thickness healing on tangentially excised second-degree burns". Chu C.S., et. al.	Electrical activation

Knowledge Finder®: Retrieved Documents Page 1 Fri Jan 12 04:47:27 2001

List Contains 1 Item.

Current Search Formulation: +LYNCH SE; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Effects of the platelet-derived growth factor/insulin-like growth factor-I combination on bone regeneration around titanium dental implants. Results of a pilot study in beagle dogs.

ARTICLE SOURCE: J Periodontol (United States), Nov 1991, 62(11) p710-6

AUTHOR(S): Lynch SE; Buser D; Hernandez RA; Weber HP; Stieh H; Fox CH; Williams RC

AUTHOR'S ADDRESS: Department of Periodontology, Harvard School of Dental Medicine, Boston, MA.

MAJOR SUBJECT HEADING(S): Bone Regeneration [drug effects]; Dental Implantation, Endosseous; Dental Implants; Insulin-Like Growth Factor I [therapeutic use]; Mandible [surgery]; Platelet-Derived Growth Factor [therapeutic use]; Titanium

MINOR SUBJECT HEADING(S): Analysis of Variance; Dogs; Drug Combinations; Gels; Insulin-Like Growth Factor I [administration & dosage]; Mandible [pathology] [physiopathology]; Methylcellulose; Pilot Projects; Placebos; Platelet-Derived Growth Factor [administration & dosage]; Recombinant Proteins; Wound Healing

INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: The purpose of this study was to evaluate the early wound healing events of bone around press-fit titanium implants inserted with and without the concurrent application of a combination of platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF-I). Nine months prior to implant placement all mandibular premolar teeth were extracted in 8 beagle dogs. Subsequently, 40 specially manufactured titanium implants with 2 transverse holes in the apical section were press fit into precise recipient sites in the dogs' mandibles. The dogs were sacrificed at 7 and 21 days following implant placement yielding 12 PDGF-B/IGF-I treated and 8 control (placebo gel or non-treated) implants for each observation period. Coded undecalcified sections were analyzed for: 1) percentage of implant surface in contact with new bone; 2) percentage of peri-implant space filled with new bone; and 3) percentage of implant hole filled with new bone. An analysis of variance was used to determine significant differences among the treatment groups. At 7 days, the percentage of bone fill in the peri-implant spaces and the percentage of implant surface in contact with new bone were both significantly increased in PDGF-B/IGF-I treated sites (P less than 0.01 for both groups). There was less than 1.5% fill of the implant holes in both treated and control sites (no significant differences). At 21 days the percentage of bone fill in the peri-implant spaces was significantly increased in the PDGF-B/IGF-I treated sites (P less than 0.01). (ABSTRACT TRUNCATED AT 250 WORDS).

MEDLINE INDEXING DATE: 199204

ISSN: 0022-3492

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 92092155

CAS REGISTRY/EC NUMBER(S): 0 (Dental Implants); 0 (Drug Combinations); 0 (Gels); 0 (Placebos); 0 (Platelet-Derived Growth Factor); 0 (Recombinant Proteins); 67763-96-6 (Insulin-Like Growth Factor I); 7440-32-6 (Titanium); 9004-67-5 (Methylcellulose)

GRANT ID NUMBER: 5T32 DE07010-DE-NIDR; K16 DE 0027501-DE-NIDR

EXHIBIT C-1

List Contains 1 Item.

Current Search Formulation: "gel delivery "

This Document Selected From: 1986 - 1995 SurgAnLine® [1996 Edition]

ARTICLE TITLE: Electrically erodible polymer gel for controlled release of drugs.

ARTICLE SOURCE: Nature (England), Nov 28 1991, 354(6351) p291-3

AUTHOR(S): Kwon IC; Bae YH; Kim SW

AUTHOR'S ADDRESS: Center for Controlled Chemical Delivery, University of Utah, Salt Lake City 84108.

MAJOR SUBJECT HEADING(S): Delayed-Action Preparations

MINOR SUBJECT HEADING(S): Acrylic Resins [chemistry]; Electric Stimulation; Hydrogen-Ion Concentration; Insulin [administration & dosage]; Oxazoles [chemistry]; Polymers [chemistry]; Polymethacrylic Acids [chemistry]; Solubility

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: New controlled drug-delivery systems are being explored to overcome the disadvantages of conventional dosage forms. For example, stimulated drug-delivery has been used to overcome the tolerance problems that occur with a constant delivery rate, to mimic the physiological pattern of hormonal concentration and to supply drugs on demand. Stimuli-sensitive polymers, which are potentially useful for pulsed drug delivery, experience changes in either their structure or their chemical properties in response to changes in environmental conditions. Environmental stimuli include temperature, pH, light (ultraviolet or visible), electric field or certain chemicals. Volume changes of stimuli-sensitive gel networks are particularly responsive to external stimuli, but swelling is slow to occur. As well as being useful in the controlled release of drugs, such systems also provide insight into intermolecular interactions. Here we report on a novel polymeric system, which rapidly changes from a solid state to solution in response to small electric currents, by disintegration of the solid polymer complex into two water-soluble polymers. We show that the modulated release of insulin, and by extension other macromolecules, can be achieved with this polymeric system.

MEDLINE INDEXING DATE: 9203

ISSN: 0028-0836

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 92065953

CAS REGISTRY/EC NUMBER(S): 0 (Acrylic Resins); 0 (Delayed-Action Preparations); 0 (Oxazoles); 0 (Polymers); 0 (Polymethacrylic Acids); 11061-68-0 (Insulin); 25087-26-7 (polymethacrylic acid); 25805-17-8 (polyethyloxazoline); 9003-01-4 (carbopol 940)

EXHIBIT C-2

List Contains 1 Item.

Current Search Formulation: +ASPENBERG P; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Dose-dependent stimulation of bone induction by basic fibroblast growth factor in rats.

ARTICLE SOURCE: Acta Orthop Scand (Denmark), Oct 1991, 62(5) p481-4

AUTHOR(S): Aspenberg P; Thorngren KG; Lohmander LS

AUTHOR'S ADDRESS: Lund University Hospital Department of Orthopedics, Sweden.

MAJOR SUBJECT HEADING(S): Bone Matrix [transplantation]; Fibroblast Growth Factor, Basic [pharmacology]; Osteogenesis [drug effects]

MINOR SUBJECT HEADING(S): Abdominal Muscles [surgery]; Bone Matrix [chemistry]; Calcium [analysis]; Dose-Response Relationship, Drug; Fibroblast Growth Factor, Basic [administration & dosage]; Rats, Inbred Strains; Rats

INDEXING CHECK TAG(S): Animal; Female; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Implantation of demineralized bone matrix in rodents elicits a series of cellular events leading to the formation of new bone inside and adjacent to the implant. This process is believed to be initiated by an inductive protein present in bone matrix, and local growth factors may further regulate the process. We have previously shown that local application of recombinant human basic fibroblast growth factor (bFGF) in a carboxymethyl cellulose gel to demineralized bone matrix implants increases the bone yield as measured by calcium content 3 weeks after implantation in rats. We now report that this increase was seen at 3 and 4 weeks, but not earlier or later. Further, the stimulatory effect was seen with doses from 3 to 75 ng per implant. A dose of 0.6 or 380 ng did not increase the bone yield, and 1,900 ng had a marked inhibitory effect. This narrow dosage optimum may reflect the complex actions of the growth factor.

MEDLINE INDEXING DATE: 199202

ISSN: 0001-6470

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 92057648

CAS REGISTRY/EC NUMBER(S): 0 (Fibroblast Growth Factor, Basic); 7440-70-2 (Calcium)

EXHIBIT C-3

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TITLE: Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells.

AUTHORS: Jackson A; Friedman S; Zhan X; Engleka KA; Forough R; Maciag T

AUTHOR AFFILIATION: Department of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, American Red Cross, Rockville, MD 20855.

SOURCE: Proc Natl Acad Sci U S A 1992 Nov 15;89(22):10691-5

CITATION IDS: PMID: 1279690 UI: 93066309

ABSTRACT: Fibroblast growth factor 1 (FGF-1) is a potent angiogenic and neurotrophic factor whose structure lacks a classical signal sequence for secretion. Although the initiation of these biological activities involves the interaction between FGF-1 and cell surface receptors, the mechanism responsible for the regulation of FGF-1 secretion is unknown. We report that murine NIH 3T3 cells transfected with a synthetic gene encoding FGF-1 secrete FGF-1 into their conditioned medium in response to heat shock. The form of FGF-1 released by NIH 3T3 cells in response to increased temperature (42 degrees C, 2 hr) in vitro is not biologically active and does not associate with either heparin or the extracellular NIH 3T3 monolayer matrix. However, it was possible to derive biologically active FGF-1 from the conditioned medium of heat-shocked NIH 3T3 cell transfectants by ammonium sulfate fractionation. The form of FGF-1 exposed by ammonium sulfate fractionation is similar in size to cytosolic FGF-1 and can bind and be eluted from immobilized heparin similarly to the recombinant human FGF-1 polypeptide. Further, the release of FGF-1 by NIH 3T3 cell transfectants in response to heat shock is reduced significantly by both actinomycin D and cycloheximide. These data indicate that increased temperature may upregulate the expression of a factor responsible for the secretion of FGF-1 as a biologically

EXHIBIT C-4

inactive complex that requires an activation step to exhibit the biological activity of the extracellular polypeptide mitogen.

MAIN MESH HEADINGS:

Fibroblast Growth Factor, Acidic/*biosynthesis
*Heat

ADDITIONAL MESH HEADINGS:

Animal
Cell Division
Culture Media, Conditioned
Cycloheximide/pharmacology
Cytosol/metabolism
Dactinomycin/pharmacology
DNA/biosynthesis
Fibroblast Growth Factor, Acidic/genetics
Fibroblast Growth Factor, Acidic/pharmacology
Fibroblast Growth Factor, Acidic/secretion
Genes, Synthetic
Immunoblotting
Kinetics
Mice
Recombinant Proteins/pharmacology
Support, U.S. Gov't, P.H.S.
Thymidine/metabolism
Transfection
Tritium
3T3 Cells
1992/11
1992/15 00:00

PUBLICATION TYPES:

JOURNAL ARTICLE

CAS REGISTRY NUMBERS:

0 (Culture Media, Conditioned)
0 (Recombinant Proteins)
10028-17-8 (Tritium)
104781-85-3 (Fibroblast Growth Factor, Acidic)
50-76-0 (Dactinomycin)
50-89-5 (Thymidine)
66-81-9 (Cycloheximide)
9007-49-2 (DNA)

LANGUAGES:

Eng

GRANT/CONTRACT ID:

HL32348/HL/NHLBI
HL44336/HL/NHLBI



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Current Search Formulation: +MARELLI D

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Cell transplantation for myocardial repair: an experimental approach.

ARTICLE SOURCE: Cell Transplant (United States), 1992, 1(6) p383-90

AUTHOR(S): Marelli D; Desrosiers C; el-Alfy M; Kao RL; Chiu RC

AUTHOR'S ADDRESS: Department of Surgery, McGill University, Montreal, Quebec, Canada.

MAJOR SUBJECT HEADING(S): Muscles [transplantation]; Myocardial Diseases [surgery]; Myocardium [pathology]; Transplantation, Heterotopic

MINOR SUBJECT HEADING(S): Cells, Cultured; Dogs; Freezing; Muscles [cytology] [physiology]; Myocardial Diseases [pathology]; Regeneration; Tissue Culture [methods]; Transplantation, Autologous; Transplantation, Heterotopic [methods] [physiology]

INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Myocardium lacks the ability to regenerate following injury. This is in contrast to skeletal muscle (SKM), in which capacity for tissue repair is attributed to the presence of satellite cells. It was hypothesized that SKM satellite cells multiplied in vitro could be used to repair injured heart muscle. Fourteen dogs underwent explantation of the anterior tibialis muscle. Satellite cells were multiplied in vitro and their nuclei were labeled with tritiated thymidine 24 h prior to implantation. The same dogs were then subjected successfully to a myocardial injury by the application of a cryoprobe. The cells were suspended in serum-free growth medium and autotransplanted within the damaged muscle. Medium without cells was injected into an adjacent site to serve as a control. Endpoints comprised histology using standard stains as well as Masson trichrome (specific for connective tissue), and radioautography. In five dogs, satellite cell isolation, culture, and implantation were technically satisfactory. In three implanted dogs, specimens were taken within 6-8 wk. There were persistence of the implantation channels in the experimental sites when compared to the controls. Macroscopically, muscle tissue completely surrounded by scar tissue could be seen. Masson trichrome staining showed homogeneous scar in the control site, but not in the test site where a patch of muscle fibres containing intercalated discs (characteristic of myocardial tissue) was observed. In two other dogs, specimens were taken at 14 wk postimplantation. Muscle tissue could not be found. These preliminary results could be consistent with the hypothesis that SKM satellite cells can form neo-myocardium within an appropriate environment. Our specimens failed to demonstrate the presence of myocyte nuclei. (ABSTRACT TRUNCATED AT 250 WORDS).

MEDLINE INDEXING DATE: 199407

ISSN: 0963-6897

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 94199205

EXHIBIT C-5

List Contains 1 Item.

Current Search Formulation: +YOUNG S; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Macrophage responsiveness to light therapy.

ARTICLE SOURCE: Lasers Surg Med (United States), 1989, 9(5) p497-505

AUTHOR(S): Young S; Bolton P; Dyson M; Harvey W; Diamantopoulos C

AUTHOR'S ADDRESS: Anatomy Department, United Medical School, Guy's Hospital, London, England.

MAJOR SUBJECT HEADING(S): Growth Substances [physiology]; Lasers [therapeutic use]; Macrophages [radiation effects]; Wound Healing [radiation effects]

MINOR SUBJECT HEADING(S): Cell Division [radiation effects]; Cell Line; Cells, Cultured; Fibroblasts [cytology] [radiation effects]; Growth Substances [secretion]; Kidney [cytology]; Macrophages [cytology] [secretion]; Mice

INDEXING CHECK TAG(S): Animal; Comparative Study; In Vitro; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Macrophages are a source of many important mediators of wound repair. It was the purpose of this study to see if light could stimulate the release of these mediators. In this study an established macrophage-like cell line (U-937) was used. The cells were exposed in culture to the following wavelengths of light: 660 nm, 820 nm, 870 nm, and 880 nm. The 820-nm source was coherent and polarised, and the others were non-coherent. Twelve hours after exposure the macrophage supernatant was removed and placed on 3T3 fibroblast cultures. Fibroblast proliferation was assessed over a 5-day period. The results showed that 660-nm, 820-nm, and 870-nm wavelengths encouraged the macrophages to release factors that stimulated fibroblast proliferation above the control levels, whereas the 880-nm wavelength either inhibited the release of these factors or encouraged the release of some inhibitory factors of fibroblast proliferation. These results suggest that light at certain wavelengths may be a useful therapeutic agent by providing a means of either stimulating or inhibiting fibroblast proliferation where necessary. At certain wavelengths coherence is not essential.

MEDLINE INDEXING DATE: 199002

ISSN: 0196-8092

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 90042969

CAS REGISTRY/EC NUMBER(S): 0 (Growth Substances)

EXHIBIT C-6

List Contains 1 Item.

Current Search Formulation: +STOMPRO BE; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Attachment of peptide growth factors to implantable collagen.

ARTICLE SOURCE: J Surg Res (United States), May 1989, 46(5) p413-21

AUTHOR(S): Stompro BE; Hansbrough JF; Boyce ST

AUTHOR'S ADDRESS: Department of Surgery, University of California, San Diego Medical Center 92103.

MAJOR SUBJECT HEADING(S): Collagen; Epidermal Growth Factor-Urogastrone; Epidermis [cytology]; Growth Substances; Heparin; Keratin

MINOR SUBJECT HEADING(S): Cell Division; Cells, Cultured; Drug Combinations; Epidermal Growth Factor-Urogastrone [pharmacology]; Growth Substances [pharmacology]; Heparin [pharmacology]; Wound Healing

INDEXING CHECK TAG(S): Support, U.S. Gov't, P.H.S.

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Ingrowth of fibrovascular tissue from the woundbed into collagen-based dermal substitutes and survival of cultured epithelium after transplantation may be enhanced by attachment of heparin binding growth factor 2 (HBGF2) and epidermal growth factor (EGF) to collagen. Biotinylation of collagen and the growth factors allows immobilization of HBGF2 and EGF by high affinity binding of tetravalent avidin. Biotinylated HBGF2 and EGF (B-GF) were exposed to complexes of biotinylated collagen (B-COL)-avidin (A) and detected with peroxidase-labeled avidin (AP) followed by chromagen formation on nitrocellulose paper. Binding of biotinylated HBGF2 and EGF was specific (*, P less than 0.05), proportional to the concentration of biotinylated collagen, and resistant to ionic (NaCl) displacement. Data are expressed as mean percentages of maximum binding +/- SEMs: (table; see text) Growth response of cultured human epidermal keratinocytes to HBGF2 (population doubling time, PDT = 0.70 population doublings (PD)/day) confirmed the retention of mitogenic activity after biotinylation (PDT = 0.80 PD/day). Specific binding of biotinylated HBGF2, EGF, or other biologically active molecules (antibiotics, NSAIDs) to implantable collagen may provide a mechanism for positive therapeutic modulation of wound healing, including repair of full-thickness skin wounds with cultured cell-collagen composite grafts.

MEDLINE INDEXING DATE: 198908

ISSN: 0022-4804

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 89237142

CAS REGISTRY/EC NUMBER(S): 0 (Drug Combinations); 0 (Fibroblast Growth Factor, Basic); 0 (Growth Substances); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 68238-35-7 (Keratin); 9005-49-6 (Heparin); 9007-34-5 (Collagen)

GRANT ID NUMBER: GM35068-GM-NIGMS

EXHIBIT C-7

List Contains 1 Item.

Current Search Formulation: +SATO T; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Bone morphogenesis of rabbit bone morphogenetic protein-bound hydroxyapatite-fibrin composite.

ARTICLE SOURCE: Clin Orthop (United States), Feb 1991, (263) p254-62

AUTHOR(S): Sato T; Kawamura M; Sato K; Iwata H; Miura T

AUTHOR'S ADDRESS: Department of Orthopaedic Surgery, Nagoya University School of Medicine, Japan.

MAJOR SUBJECT HEADING(S): Composite Resins [therapeutic use]; Fibrin [therapeutic use]; Growth Substances [therapeutic use]; Osteogenesis [drug effects]; Proteins [therapeutic use]

MINOR SUBJECT HEADING(S): Bone and Bones [drug effects]; Composite Resins [pharmacology]; Fibrin [pharmacology]; Growth Substances [pharmacology]; Hydroxyapatites [therapeutic use]; Proteins [pharmacology]; Rabbits

INDEXING CHECK TAG(S): Animal

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Hydroxyapatite (HAP) and fibrin have been implanted in patients and observed to be well tolerated in orthotopic sites.

This is a report on a composite of HAP, fibrin, and rabbit bone morphogenetic protein and insoluble noncollagenous protein (BMP-iNCP). Drill holes in the femoral condyles of rabbits were packed with granulated HAP (200 mg), fibrin (0.3 ml), BMP-iNCP (5 mg), or various combinations of the two. The fibrin consisted mainly of sterilized human fibrinogen and thrombin, and BMP-iNCP was prepared from demineralized rabbit cortical bone. New bone formation was observed at one, two, four, and eight weeks after implantation. The BMP-iNCP augmented new bone formation in rabbit femoral condyles. Fibrin made the composite easier to manipulate and did not inhibit osteogenesis at any period. The composites of HAP with BMP-iNCP and of HAP with BMP-iNCP and fibrin produced higher yields of new bone than fibrin alone or HAP alone.

MEDLINE INDEXING DATE: 199105

ISSN: 0009-921X

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 91130138

CAS REGISTRY/EC NUMBER(S): 0 (Bone Morphogenetic Proteins); 0 (Composite Resins); 0 (Growth Substances); 0 (Hydroxyapatites); 1306-06-5 (Durapatite); 9001-31-4 (Fibrin)

EXHIBIT C-8

List Contains 1 Item.

Current Search Formulation: +HOCKEL M; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Angiotropin treatment prevents flap necrosis and enhances dermal regeneration in rabbits.

ARTICLE SOURCE: Arch Surg (United States), Jun 1989, 124(6) p693-8

AUTHOR(S): Hockel M; Burke JF

AUTHOR'S ADDRESS: Universitätsfrauenklinik Mainz, West Germany.

MAJOR SUBJECT HEADING(S): Angiogenesis Factor [pharmacology]; Growth Substances [pharmacology]; Necrosis [prevention & control]; Skin [pathology]; Surgical Flaps

MINOR SUBJECT HEADING(S): Angiogenesis Factor [administration & dosage]; Graft Survival; Injections, Intradermal; Rabbits; Skin [blood supply]; Wound Healing

INDEXING CHECK TAG(S): Animal; Female; Male

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Angiotropin is a potent angiogenesis factor isolated from the serum-free media of cultured, lectin-activated peripheral monocytes. In vitro, the purified substance stimulates migration, phenotypic differentiation, and tube formation, but not proliferation of capillary endothelial cells. When injected intradermally, angiotropin induces, in dose-dependent fashion, angiogenesis associated with skin hyperplasia. We have developed a flap model with insufficient blood supply and a model for contraction-free defect healing in rabbit skin. We show that (1) local pretreatment with angiotropin can prevent flap necrosis and (2) dermal regeneration after wounding can be augmented by angiotropin. From these results, we conclude that angiotropin might be of use as an adjuvant to healing in surgery.

MEDLINE INDEXING DATE: 198909

ISSN: 0004-0010

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 89272615

CAS REGISTRY/EC NUMBER(S): 0 (Angiogenesis Factor); 0 (Growth Substances)

EXHIBIT C-9

List Contains 1 Item.

Current Search Formulation: +KHOURI RK; + 1991 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Tissue transformation into bone in vivo. A potential practical application.

ARTICLE SOURCE: JAMA (United States), Oct 9 1991, 266(14) p1953-5

AUTHOR(S): Khouri RK; Koudsi B; Reddi H

AUTHOR'S ADDRESS: Department of Surgery, Washington University School of Medicine, St Louis, Mo. 63110.

MAJOR SUBJECT HEADING(S): Bone and Bones [physiopathology]; Glycoproteins [administration & dosage]; Growth Substances [administration & dosage]; Muscles [transplantation]; Osteogenesis; Proteins [administration & dosage]

MINOR SUBJECT HEADING(S): Bone Matrix; Bone and Bones [surgery]; Injections; Osteogenesis [drug effects]; Rats, Inbred Lew; Rats; Surgical Flaps; Tissue Transplantation [methods]

INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: The transformation of mesenchymal tissue, such as muscle, into cartilage and bone can be induced by the recently purified osteoinductive factor, osteogenin, and by its parent substratum, demineralized bone matrix. We investigated the possibility of transforming readily available muscle flaps into vascularized bone grafts of various shapes that could be used as skeletal replacement parts. In a rat experimental model, thigh adductor muscle island flaps were placed inside bivalved silicone rubber molds. Prior to closure of the mold, 18 flaps were injected with osteogenin and coated with demineralized bone matrix. Five flaps served as controls and were injected with the vehicle only, and not coated with demineralized bone matrix. The molds were implanted subcutaneously in the rats' flanks and reopened 10 days later. The control flaps consisted of intact muscle without any evidence of tissue transformation, whereas the flaps treated with osteogenin and demineralized bone matrix were entirely transformed into cancellous bone that matched the exact shape of the mold. Using tissue transformation, we were able to generate in vivo, autogenous, well-perfused bones in the shapes of femoral heads and mandibles.

MEDLINE INDEXING DATE: 199112

ISSN: 0098-7484

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 91374707

CAS REGISTRY/EC NUMBER(S): 0 (osteogenin); 0 (osteoinductive factor); 0 (Glycoproteins); 0 (Growth Substances)

GRANT ID NUMBER: 22-3335 44901A

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TITLE: An experimental evaluation of microcapsules for arterial chemoembolization.

AUTHORS: Bechtel W; Wright KC; Wallace S; Mosier B; Mosier D; Mir S; Kudo S

SOURCE: Radiology 1986 Dec;161(3):601-4

CITATION IDS: PMID: 2947261 UI: 87068344

ABSTRACT: Microcapsules, 106 micron (range, 50-350 micron), of different capsular materials (monoglyceride, monodiglyceride, natural wax, cellulose polymer, or lactic acid polymer) with and without floxuridine (2'-deoxy-5-fluorouridine, FUDR) were intraarterially injected into dog kidneys. The drug-release characteristics of the microcapsules, as determined by analysis of renal and systemic venous blood samples over a 6-hour period, were uniphasic or multiphasic depending on the capsular material. Histologic changes of varying degrees were noted in all kidneys embolized except for those subjected to capsules of the cellulose polymer. The most striking changes were produced by the lactide polymer capsules. The potential applications of microencapsulated chemotherapeutic agents in intraarterial transcatheter treatment of cancer are discussed.

MAIN MESH HEADINGS: Antineoplastic Agents/*administration & dosage
*Embolization, Therapeutic

ADDITIONAL MESH HEADINGS: Animal
Antineoplastic Agents/blood
Capsules
Combined Modality Therapy
Dogs
Floxuridine/administration & dosage
Floxuridine/blood
Renal Artery
Support, Non-U.S. Gov't
Support, U.S. Gov't, P.H.S.

EXHIBIT C-11

10/26/01

List Contains 1 Item.

Current Search Formulation: +SPRECHER DL; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Histopathologic examination of material from angioplasty balloon catheters used in vivo in human coronary arteries.

ARTICLE SOURCE: Atherosclerosis (Netherlands), Feb 1989, 75(2-3) p237-44

AUTHOR(S): Sprecher DL; Mikat EM; Stack R; Sutherland K; Schneider J; Bashore T; Hackel DB

AUTHOR'S ADDRESS: University of Cincinnati Medical Center, Department of Pathology, OH 45267-0529.

MAJOR SUBJECT HEADING(S): Angioplasty, Balloon; Arteriosclerosis [pathology]; Atherosclerosis [pathology]; Coronary Vessels [pathology]; Specimen Handling [methods]

MINOR SUBJECT HEADING(S): Adult; Aged; Angina Pectoris [therapy]; Coronary Vessels [cytology]; Middle Age; Myocardial Infarction [therapy]

INDEXING CHECK TAG(S): Female; Human; Male; Support, U.S. Gov't, P.H.S.

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Reports on vascular pathology post-PTCA in both human and animal coronary vessels have revealed medial and intimal cracks and tears, thrombus formation, platelet accumulation, and loss of endothelial cells. The extent and type of damage can currently be assessed in vivo at the macro level by means of coronary artery angiography. However, this technique cannot define vessel wall characteristics at the cellular level. Our hypothesis is that vessel wall material may adhere to the balloon and thus provide a source for coronary artery cytological investigation in vivo. Ten balloon catheters were evaluated to discern any material which was dislodged from the coronary artery and which remained attached to the balloon catheter or guide wire. Our results indicate that angioplasty catheter balloons frequently have adherent collagen, endothelial cells, organized thrombus, and plaque with obvious cholesterol clefts, that can be retrieved and examined histologically. We conclude that material is often dislodged from the plaque during PTCA. In addition, plaque material removed by the balloon catheter offers an unusual opportunity to analyze the morphologic characteristics of cells from the human coronary artery in vivo.

MEDLINE INDEXING DATE: 198908

ISSN: 0021-9150

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 89228141

GRANT ID NUMBER: HLB 17670

EXHIBIT C-12

List Contains 1 Item.

Current Search Formulation: +JULLIEN P; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF.

ARTICLE SOURCE: Int J Cancer (United States), May 15 1989, 43(5) p886-91

AUTHOR(S): Jullien P; Berg TM; Lawrence DA

AUTHOR'S ADDRESS: Unite 532 CNRS, Institut Curie-Biologie, Orsay, France.

MAJOR SUBJECT HEADING(S): Cell Transformation, Neoplastic; Epidermal Growth Factor-Urogastrone [pharmacology];

Transforming Growth Factors [biosynthesis]

MINOR SUBJECT HEADING(S): Agar; Blood; Cell Division [drug effects]; Cell Line; Culture Media; Hydrogen-Ion Concentration;

Lactates [pharmacology]; Mice; Transforming Growth Factors [pharmacology]

INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Transient (about 2 hr) acidification to approx. pH 5.0 of agar-gelled overlayers containing untransformed NRK-49F or KiMSV-transformed NRK-49F cells in the presence of fetal calf serum or crude 49F-cell conditioned medium, as sources of latent TGF-beta, elicited EGF-dependent colony formation of 49F cells and inhibited spontaneous growth of transformed cells. Pure, active TGF-beta (porcine, type I) had the same effects on these respective cell types, suggesting that the above results were due to activation of latent TGF-beta in the transiently acidic cellular environment. Similar acidifications in the absence of a source of latent TGF-beta enhanced the positive growth response of 49F and AKR-2B cells to EGF and active TGF-beta and also the negative growth response of KiMSV-transformed 49F cells to active TGF-beta. These results are compatible with the idea that acidic cellular environments, particularly in tumor tissues, are conducive to activation of latent TGF-beta, perhaps in conjunction with other activating mechanisms, and to an enhanced response to some growth factors. However, the heterogeneity of cell populations within tumoral masses presents an obstacle to a clear understanding of the consequences of such activation.

MEDLINE INDEXING DATE: 198908

ISSN: 0020-7136

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 89233486

CAS REGISTRY/EC NUMBER(S): 0 (Culture Media); 0 (Lactates); 50-21-5 (Lactic Acid); 62229-50-9 (Epidermal Growth Factor-Urogastrone); 76057-06-2 (Transforming Growth Factors); 9002-18-0 (Agar)

EXHIBIT C-13

List Contains 1 Item.

Current Search Formulation: +MERRILEES MJ; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Endothelial cell stimulation of smooth muscle glycosaminoglycan synthesis can be accounted for by transforming growth factor beta activity.

ARTICLE SOURCE: Atherosclerosis (Netherlands), Apr 1990, 81(3) p255-65

AUTHOR(S): Merrilees MJ; Scott L

AUTHOR'S ADDRESS: Department of Anatomy, School of Medicine, University of Auckland, New Zealand.

MAJOR SUBJECT HEADING(S): Endothelium, Vascular [physiology]; Glycosaminoglycans [biosynthesis]; Muscle, Smooth, Vascular [metabolism]; Transforming Growth Factors [physiology]

MINOR SUBJECT HEADING(S): Cells, Cultured; Endothelium, Vascular [metabolism]; Sulfhydryl Compounds [pharmacology]; Swine; Transforming Growth Factors [metabolism]; Trypsin [pharmacology]

INDEXING CHECK TAG(S): Animal; Support, Non-U.S. Gov't

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Endothelial cell conditioned medium (ECCM) contains a factor which markedly stimulates smooth muscle cell (SMC) glycosaminoglycan (GAG) synthesis. We report here that the factor responsible is transforming growth factor beta (TGF-beta) as assessed by (1) protease and thiol sensitivity, (2) heat and acid enhancement of ECCM activity, and (3) neutralisation of ECCM activity by anti-TGF-beta-immunoglobulin. Anti-TGF-beta-neutralisation was effective against increases in both sulphated and non-sulphated GAG. Previous studies showed that ECCM from EC of varying densities stimulated individual GAG to varying degrees. ECCM from low density EC preferentially stimulated hyaluronic acid (HA) whereas ECCM from intermediate and high density cultures stimulated increasing amounts of sulphated GAG. Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern. Exposure of SMC to varying concentrations of TGF-beta produced a similar pattern of response. Very low amounts of TGF-beta (less than 10-500 pg/10 cells) stimulated a marked and significant increase in HA synthesis. Increase in chondroitin sulphate 4/6 was most marked at TGF-beta levels from 500-1000 pg/10(6) cells. At levels above 1000 pg/10(6) cells both HA and sulphated GAG synthesis decreased but still remained elevated above controls. These findings indicate that TGF-beta alone can account for the changes in SMC GAG synthesis stimulated by ECCM. It was also found, however, that heat-treated SMC conditioned medium stimulated SMC GAG synthesis, thus SMC may contribute to the control of their own GAG synthesis through autocrine TGF-beta activity.

MEDLINE INDEXING DATE: 199009

ISSN: 0021-9150

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 90274739

CAS REGISTRY/EC NUMBER(S): EC 3.4.21.4 (Trypsin); 0 (Glycosaminoglycans); 0 (Sulfhydryl Compounds); 76057-06-2 (Transforming Growth Factors)

EXHIBIT C-14

List Contains 1 Item.

Current Search Formulation: +YOUNG SR; + 1990 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Macrophage responsiveness to therapeutic ultrasound.

ARTICLE SOURCE: Ultrasound Med Biol (England), 1990, 16(8) p809-16

AUTHOR(S): Young SR; Dyson M

AUTHOR'S ADDRESS: Department of Anatomy, United Medical School, Guy's Hospital, London, England.

MAJOR SUBJECT HEADING(S): Macrophages [cytology]; Ultrasonic Therapy

MINOR SUBJECT HEADING(S): Cell Count; Cell Division; Cell Line; Cell Survival; Fibroblasts [metabolism]; Growth Substances [biosynthesis]; Macrophages [metabolism]

INDEXING CHECK TAG(S): Animal; Human

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Macrophages are a source of many important growth factors which can act as wound mediators during tissue repair. The aim of this work was to find out if levels of ultrasound which accelerate repair could stimulate the release of fibroblast mitogenic factors from an established macrophage-like cell line (U937). The U937 cells were exposed in vitro to continuous ultrasound at a space average, temporal average intensity of 0.5 W/cm² at either 0.75 MHz or 3.0 MHz, for 5 min. The macrophage-conditioned medium was removed either 30 min or 12 h after exposure, and placed on 3T3 fibroblast cultures. Fibroblast proliferation (defined here as increase in cell number) was assessed over a 5-day period. The results showed that 0.75 MHz ultrasound appeared to be effective in liberating preformed fibroblast affecting substances from the U937 cells, possibly by producing permeability changes, whereas 3.0 MHz ultrasound appeared to stimulate the cell's ability to synthesize and secrete fibroblast mitogenic factors.

MEDLINE INDEXING DATE: 199109

ISSN: 0301-5629

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 91247100

CAS REGISTRY/EC NUMBER(S): 0 (fibroblast-activating factor); 0 (Growth Substances)

EXHIBIT C-15

List Contains 1 Item.

Current Search Formulation: +SHULTZ PJ; + 1989 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Mitogenic signals for thrombin in mesangial cells: regulation of phospholipase C and PDGF genes.

ARTICLE SOURCE: Am J Physiol (United States), Sep 1989, 257(3 Pt 2) pF366-74

AUTHOR(S): Shultz PJ; Knauss TC; Mene P; Abboud HE

AUTHOR'S ADDRESS: Department of Medicine, Veterans Administration Medical Center, Cleveland, Ohio.

MAJOR SUBJECT HEADING(S): Gene Expression Regulation; Glomerular Mesangium [physiology]; Mitogens [physiology]; Phospholipase C [genetics]; Platelet-Derived Growth Factor [genetics]; Thrombin [physiology]

MINOR SUBJECT HEADING(S): Calcium [metabolism]; Cytosol [metabolism]; Gene Expression Regulation [drug effects]; Glomerular Mesangium [cytology] [metabolism]; Mitogens [pharmacology]; Phosphatidylinositols [metabolism]; Proteins [metabolism]; RNA, Messenger [metabolism]; Thrombin [pharmacology]

INDEXING CHECK TAG(S): Human; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: Thrombin is a proteolytic enzyme of diverse biological activities, which is produced during activation of the coagulation pathway. In addition, thrombin is a mitogen for fibroblasts and endothelial cells. Intraglomerular thrombosis and cell proliferation are common pathological features of several glomerular diseases. We studied the effect of thrombin on deoxyribonucleic acid (DNA) synthesis in cultured human mesangial cells and explored mechanisms of signal transduction involved. Bovine and human thrombin caused dose-dependent increases in DNA synthesis, inositol trisphosphate, and cytosolic calcium $[(Ca^{2+})_i]$. A threefold increase in inositol-3-trisphosphate (IP3) levels was observed as early as 10 s after the addition of thrombin, whereas increases in $(Ca^{2+})_i$ occurred within 5-10 s and declined rapidly. Stimulation of mesangial cells by thrombin resulted in induction of messenger ribonucleic acids (mRNAs) encoding platelet-derived growth factor (PDGF) A- and B-chains. This was associated with an enhanced secretion of PDGF-like protein. These data provide mechanisms by which thrombin may regulate mesangial cell function in disease states.

MEDLINE INDEXING DATE: 198912

ISSN: 0002-9513

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 89390640

CAS REGISTRY/EC NUMBER(S): EC 3.1.4.3 (Phospholipase C); EC 3.4.21.5 (Thrombin); 0 (Mitogens); 0 (Phosphatidylinositols); 0 (Platelet-Derived Growth Factor); 0 (RNA, Messenger); 7440-70-2 (Calcium)

GRANT ID NUMBER: DK-33665-DK-NIDDK; DK-07470-DK-NIDDK

EXHIBIT C-16

Knowledge Finder®: Retrieved Documents Page 1 Wed Jan 24 12:39:51 2001

List Contains 1 Item.

Current Search Formulation: "electrical stimulation of growth"; + 1989 - All Articles; + 1990 - All Articles; + 1991 - All Articles; + 1992 - All Articles; + 1993 - All Articles

This Document Selected From: 1989 - 1998 SurgAnLine® [1999 Edition]

ARTICLE TITLE: Weak direct current accelerates split-thickness graft healing on tangentially excised second-degree burns.

ARTICLE SOURCE: J Burn Care Rehabil (United States), Jul-Aug 1991, 12(4) p285-93

AUTHOR(S): Chu CS; McManus AT; Okerberg CV; Mason AD Jr; Pruitt BA Jr

AUTHOR'S ADDRESS: Library Branch, United States Army Institute of Surgical Research, Fort Sam Houston, TX 78234-5012.

MAJOR SUBJECT HEADING(S): Burns [physiopathology]; Electric Stimulation Therapy; Skin Transplantation; Wound Healing [physiology]

MINOR SUBJECT HEADING(S): Burns [pathology] [surgery]; Cell Division; Guinea Pigs; Skin Transplantation [pathology]; Skin [pathology]; Transplantation, Autologous

INDEXING CHECK TAG(S): Animal; Male

PUBLICATION TYPE: JOURNAL ARTICLE

ABSTRACT: We have examined the effects of direct current (DC) conducted through silver-nylon dressings on the healing time and morphologic maturation of split-thickness grafts placed on tangentially excised deep partial-thickness burn wounds. Male guinea pigs (n = 120) were used as the experimental hosts. The DC-treated animals required 2 days for complete revascularization of their grafts; control animals required 7 days (p less than 0.01). The DC-treated animals had increased epithelial proliferation at the graft-wound interface as compared with controls (p less than 0.01). Grafts from DC-treated animals were firmly adherent within 4 days, whereas graft adherence in controls was weak before 7 days after grafting. At 3 months after grafting, control animal grafts had mild contraction with moderate hair loss and thick subepidermal fibrosis; the grafts in DC-treated animals expanded with the growth of the animals and had abundant hair growth and significantly reduced dermal fibrosis (p less than 0.01).

MEDLINE INDEXING DATE: 199202

ISSN: 0273-8481

LANGUAGE: English

UNIQUE NLM IDENTIFIER: 92042249

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia

SERIAL NO.: 09/064,000

FILED: April 21, 1998

FOR: METHOD AND APPARATUS
FOR INSTALLATION OF
DENTAL IMPLANT

EXAMINER: Nicholas D. Lucchesi

GROUP ART UT

REC'D IN MAIL
(ORIGINAL?)

Keep w/ Decl. as
filed per GKW

DECLARATION OF G. ROBERT MEGER, M.D.

I G. Robert Meger declare as follows:

1. I have offices at 3333 East Camelback Road, Phoenix, Arizona 85018.
2. My Curriculum Vitae is attached hereto as Exhibit A.
3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter "235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit B. I understand that the same disclosures are contained in above patent Application Serial No. 09/064,000.
4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and resulting soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection,

through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. The materials included in Exhibit C of this Declaration illustrate that the techniques set forth in above Paragraph 4 were well known to those skilled in the medical arts prior to July 2, 1993. It is my opinion that one skilled in the medical arts armed with such knowledge would have been able to practice the invention(s) described at column 14, lines 4-61 and column 21, lines 1-26 of the '235 patent without need for resorting to undue experimentation.
6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/13/01

G. Robert Meger
G. Robert Meger

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CURRICULUM VITAE

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EVIDENCE APPENDIX

ITEM NO. 20

**Strauer et al. 2002 publication in Circulation entitled,
“Repair of Infarcted Myocardium by Autologous Intracoronary
Mononuclear Bone Marrow Cell Transplantation in Humans”
cited by Appellant as Reference ABQ in the Third Supplemental
Information Disclosure Statement filed May 27, 2003**

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

Received August 2, 2002; accepted August 2, 2002.

From the Department of Medicine, Division of Cardiology (B.E.S., M.B., T.Z., M.K.) and Institute for Transplantation Diagnostics and Cell Therapeutics (A.H., R.V.S., G.K., P.W.), Heinrich-Heine-University of Düsseldorf, Germany.

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DOI: 10.1161/01.CIR.0000034046.87607.1C

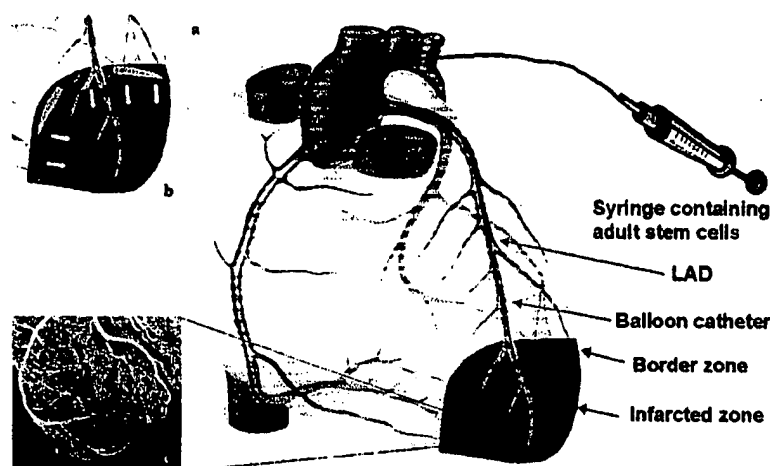


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery (n=4), left circumflex coronary artery (n=3), or right coronary artery (n=13). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty (n=20) and subsequent stent implantation (n=19). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients (n=10). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7 ; this consisted of $0.65 \pm 0.4\%$ AC133-positive cells and $2.1 \pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ⁷)	2.8±2.2

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index $P_{\text{wall}}/\text{ESV}$ was calculated by dividing LV systolic pressure (P_{wall}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11–14,18,20–23} Moreover, trans-endothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24–26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells ($\leq 1\%$), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

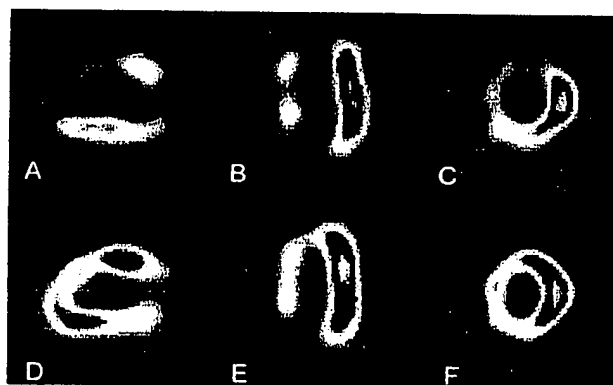


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ²⁰¹thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m ²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P _{sys} /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
²⁰¹ Thallium scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells⁸⁻¹³; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of *in vitro* amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈ 200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility ($P_{1,2}/ESV$ and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

References

- Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction: experimental observations and clinical implications. *Circulation*. 1990;81:1161-1172.
- Ertl G, Gaudron P, Hu K. Ventricular remodeling after myocardial infarction: experimental and clinical studies. *Basic Res Cardiol*. 1993;88:125-137.
- Quaini F, Urbanek K, Beltrami AP, et al. Chimerism of the transplanted heart. *N Engl J Med*. 2002;346:5-15.
- Leor J, Patterson M, Quinones MJ, et al. Transplantation of fetal myocardial tissue into infarcted myocardium of rat: a potential method for repair of infarcted myocardium? *Circulation*. 1996;94(suppl II):332-336.
- Murry CE, Wiseman RW, Schwartz SM, et al. Skeletal myoblast transplantation for repair of myocardial necrosis. *J Clin Invest*. 1996;98:2512-2523.
- Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med*. 1998;4:929-933.
- Tomita S, Li RK, Weisel RD, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation*. 1999;100(suppl II):247-256.
- Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? *Cell*. 2001;105:829-841.
- Krause DS, Theise ND, Collector ML, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*. 2001;105:369-377.
- Goodell MA, Jackson KA, Majka SM, et al. Stem cell plasticity in muscle and bone marrow. *Ann N Y Acad Sci*. 2001;938:208-218.
- Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-705.
- Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A*. 2001;98:10344-10349.
- Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430-436.
- Tomita S, Mickle DA, Weisel RD, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg*. 2002;123:1132-1135.
- Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. *Lancet*. 2001;357:279-280.
- Hamano K, Nishida M, Hirata K, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J*. 2001;65:845-847.
- Strauer BE, Brehm M, Zeus T, et al. Myocardial regeneration after intracoronary transplantation of human autologous stem cells following acute myocardial infarction. *Dtsch med Wschr*. 2001;126:932-938.
- Wang JS, Shum-Tim D, Chedrawy E, et al. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg*. 2001;122:699-705.
- Sheehan FH, Bolson EL, Dodge HT, et al. Advantages and applications of the Centerline method for characterizing regional ventricular function. *Circulation*. 1986;74:293-305.
- Sussman M. Cardiovascular biology: hearts and bones. *Nature*. 2001;410:640-641.
- Toma C, Pittenger MF, Cahill KS, et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-98.
- Kamihata H, Matsubara H, Nishie T, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation*. 2001;104:1046-1052.
- Ferrari G, Cusella-De Angelis G, Coletta M, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*. 1998;279:1528-1530.
- Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation*. 2001;103:634-637.
- Robinson SW, Cho PW, Levitsky H, et al. Arterial delivery of genetically labelled skeletal myoblasts to the murine heart: long-term survival and phenotypic modification of implanted myoblasts. *Cell Transplant*. 1996;5:77-91.
- Bittner RE, Schofer C, Weipoltshammer K, et al. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat Embryol (Berl)*. 1999;199:391-396.
- Jiang Y, Jahagirdar B, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;20:1-12.
- Reyes M, Lund T, Lenvik T, et al. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood*. 2001;98:2615-2625.
- Condorelli G, Borello U, De Angelis L, et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardial regeneration. *Proc Natl Acad Sci U S A*. 2001;98:10733-10738.
- Szilyassy SJ, Bass MJ, Van Zant G, et al. Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood*. 1999;93:1557-1566.
- Gregg DE, Fisher LC. Blood supply to the heart. In: *Handbook of Physiology*. Vol 2. Washington, DC: American Physiological Society; 1963:chap 44, 1517-1584.
- Strauer BE. Myocardial oxygen consumption in chronic heart disease: role of wall stress, hypertrophy and coronary reserve. *Am J Cardiol*. 1979;43:730-740.
- Toma C, Pittenger MF, Byrne BJ, et al. Adult human mesenchymal stem cells differentiate to a striated muscle phenotype following arterial delivery to the murine heart. *Circulation*. 2000;102(suppl II):II-683. Abstract.
- Hendrikx PJ, Martens CM, Hagenbeek A, et al. Homing of fluorescently labeled murine hematopoietic stem cells. *Exp Hematol*. 1996;24:129-140.
- Ren G, Michael LH, Entman ML, et al. Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem*. 2002;50:71-79.
- Li RK, Mickle DA, Weisel RD, et al. Optimal time for cardiomyocyte transplantation to maximize myocardial function after left ventricular injury. *Ann Thorac Surg*. 2001;72:1957-1963.
- Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002;53:31-47.
- Allgöwer M. *The Cellular Basis of Wound Repair*. Springfield, Ill: Charles C. Thomas; 1956.
- Xie Y, Zhou T, Shen W, et al. Soluble cell adhesion molecules in patients with acute coronary syndrome. *Chin Med J*. 2000;113:286-288.
- Soeki T, Tamura Y, Shinohara H, et al. Serial changes in serum VEGF and HGF in patients with acute myocardial infarction. *Cardiology*. 2000;93:168-174.
- Sheiban I, Frangasso G, Rosano GMC, et al. Time course and determinants of left ventricular function recovery after primary angioplasty in patients with acute myocardial infarction. *J Am Coll Cardiol*. 2001;38:464-471.

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